DEVELOPMENT AND VALIDATION OF A THREE COMPONENT CAPSULE FORMULATION CONTAINING MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE BY UV - SPECTROPHOTOMETRY AND HPTLC

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University

Chennai - 600 032

In partial fulfillment for the award of Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

Submitted by

DOGIPARTI MANISAIKUMAR

Register No. 26106122

Under the Guidance of

Mr. K. ANANDAKUMAR, M. Pharm.

Associate Professor.

Department of Pharmaceutical Analysis

ADHIPARASAKTHI COLLEGE OF PHARMACY

(Accredited by "NAAC" with a CGPA of 2.74 on a four point scale at "B" Grade)

MELMARUVATHUR - 603 319

MAY 2012

CERTIFICATE

This is to certify that the research work entitled "**DEVELOPMENT AND VALIDATION OF A THREE COMPONENT CAPSULE FORMULATION CONTAINING MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE BY UV - SPECTROPHOTOMETRY AND HPTLC"** is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment for the award of the Degree of the **MASTER OF PHARMACY** (Pharmaceutical Analysis) was carried out by **DOGIPARTI MANISAIKUMAR (Register No. 26106122)** in the Department of Pharmaceutical Analysis under my direct guidance and supervision during the academic year 2011 –2012.

Place : Melmaruvathur

Date :

Mr. K. ANANDAKUMAR, M. Pharm.,
Associate professor,
Department of Pharmaceutical Analysis,
Adhiparasakthi College of Pharmacy,
Melmaruvathur - 603 319.

CERTIFICATE

This is to certify that the dissertation entitled "**DEVELOPMENT AND VALIDATION OF A THREE COMPONENT CAPSULE FORMULATION CONTAINING MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE BY UV - SPECTROPHOTOMETRY AND HPTLC"** is the bonafide research work carried out by **DOGIPARTI MANISAIKUMAR** (Register No. 26106122) in the Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, Melmaruvathur which is affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai under the guidance of **Mr. K. ANANDAKUMAR, M. Pharm.,** Associate professor, Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, during the academic year 2011 – 2012.

Place: Melmaruvathur

Date:

Prof. (Dr.). T. VETRICHELVAN, M. Pharm., Ph.D.,
Principal & Head,
Department of Pharmaceutical Analysis,
Adhiparasakthi College of Pharmacy,
Melmaruvathur - 603 319.

ACKNOWLEDGEMENT

First and foremost I wish to express my deep sense of gratitude to His Holiness **ARULTHIRU AMMA** for his ever blessing in each step of study. I am grateful to **THIRUMATHI LAKSHMI BANGARU ADIGALAR**, Vice President, ACMEC trust, Melmaruvathur for having given me an opportunity and encouragement all the way in completing the study.

With great respect and honor, I extent my thanks to our Managing trustee **Mr**. **B. ANBALAGAN**, Adhiparasakthi Hospital and Research Institute, Melmaruvathur for his excellence in providing skillful and compassionate spirit of unstinted support to our department for carrying out the research work.

I received initiation and inspiration to undergo experimental investigation in modern analytical methods entitled as "**DEVELOPMENT AND VALIDATION OF A THREE COMPONENT CAPSULE FORMULATION CONTAINING MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE BY UV - SPECTROPHOTOMETRY AND HPTLC"** to this extent, I wish to sincerely record my deepest gratitude and thanks to **Mr. K. ANANDAKUMAR, M. Pharm.,** Associate Professor, Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, Melmaruvathur for his kind cooperation and initiative guidance, constructive criticism with enthusiastic encouragement which enabling me to complete this project work successfully.

On a personal note I wish to avail my deep sense of thanks to **Prof. (Dr.). T. VETRICHELVAN, M. Pharm., Ph.D.,** Principal, Adhiparasakthi

College of Pharmacy, Melmaruvathur for all the support offered by him during the period of my entire dissertation work.

I have great pleasure in express my sincere heartfelt thanks to **Dr. D. NAGAVALLI, M. Pharm., Ph.D.** Professor and **Mrs. G. ABIRAMI, M. Pharm.,** Assistant Professor, Department of Pharmaceutical Analysis, for encouragement and support for the successful completion of this work.

My sincere thanks to our lab technicians **Mr. M. GOMATHI SHANKAR, D. Pharm., Mrs. S. KARPAGAVALLI, D. Pharm.,** and **Mrs. N.THATCHAYANI, D. Pharm.,** for their kind help throughout this work.

My special thanks to our librarian **Mr. M. SURESH, M.L.I.S**., for providing all reference books to make this project a great success.

My genuine thanks to **Mrs. PRATHIMA MATHUR,** Managing Director, **Pharma Information Center,** Chennai for serving me in the literature survey.

I am grateful to my **Classmates** and **Roommates** for their pleasant working atmosphere and discussion during the work. A special thanks to my ever loving **College staffs, Friends, Seniors and Juniors** for their kind support during my work.

Last but not least, with my whole heart, I would like to express my thanks to my father **Mr. D. SUBRAMANAYAM**, my mother **Mrs. D. RAJARAJESWARI**, my brother **Mr. D. SUDHEER** and my sister **Mrs. SUPRAJA** for their encouragement and support which were a tower of strength during the entire course of work.

DOGIPARTI MANISAI KUMAR

Dedicated to

My

Parents and

friends

CONTENTS

TABLE No.	SUBJECT
1.	SOLUBILITY PROFILE OF MONTELUKAST SODIUM IN POLAR AND NON POLAR SOLVENTS
2.	SOLUBILITY PROFILE OF LEVOCETIRIZINE DIHYDROCHLORIDE IN POLAR AND NON POLAR SOLVENTS
3.	SOLUBILITY PROFILE OF AMBROXOL HYDROCHLORIDE IN POLAR AND NON POLAR SOLVENTS
4.	OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM AT 345 nm BY ABSORBANCE CORRECTION METHOD
5.	OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM AND AMBROXOL HYDROCHLORIDE AT 307 nm BY ABSORBANCE CORRECTION METHOD
6.	OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE AT 230 nm BY ABSORBANCE CORRECTION METHOD
7.	QUANTIFICATION OF FORMULATION (RENEA) BY ABSORBANCE CORRECTION METHOD
8.	INTRADAY AND INTERDAY ANALYSIS OF FORMULATION (RENEA) BY ABSORBANCE CORRECTION METHOD
9.	RUGGEDNESS STUDY OF FORMULATION (RENEA) BY ABSORBANCE CORRECTION METHOD (DIFFERENT ANALYST)
10.	RUGGEDNESS STUDY OF FORMULATION (RENEA) BY ABSORBANCE CORRECTION METHOD (DIFFERENT INSTRUMENT)
11.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY ABSORBANCE CORRECTION METHOD (80 % RECOVERY)
12.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY ABSORBANCE CORRECTION METHOD (100 % RECOVERY)
13.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY ABSORBANCE CORRECTION METHOD (120 % RECOVERY)
14.	OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM AT 365.5 nm BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY
15.	OPTICAL CHARACTERISTICS OF AMBROXOL HYDROCHLORIDE AT 256.5 nm BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY

16.	OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM AND LEVOCETIRIZINE HYDROCHLORIDE AT 248 nm BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY
17.	QUANTIFICATION OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY
18.	INTRADAY AND INTERDAY ANALYSIS OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY
19.	RUGGEDNESS STUDY OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY (DIFFERENT ANALYST)
20.	RUGGEDNESS STUDY OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY (DIFFERENT INSTRUMENT)
21.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY (80 % RECOVERY)
22.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY (100 % RECOVERY)
23.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY (120 % RECOVERY)
24.	OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE BY HPTLC
25.	QUANTIFICATION OF FORMULATION - RENEA BY HPTLC
26.	INTRADAY AND INTERDAY ANALYSIS OF FORMULATION (RENEA) BY HPTLC
27.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY HPTLC (80 % RECOVERY)
28.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY HPTLC (100 % RECOVERY)
29.	RECOVERY ANALYSIS OF FORMULATION – RENEA BY HPTLC (120 % RECOVERY)

LIST OF ABBREVIATIONS USED

%	-	Percentage
% RSD	-	Percentage Relative Standard Deviation
µ	-	Micron
µl	-	Microlitre
°C	-	Degree Celsius
gm	-	Grams
ICH	-	International Conference on Harmonisation
IR	-	Infra Red
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
mg/ tab	-	Milligram Per tablet
min	-	Minute
ml	-	Millilitre
ml/ min	-	Millilitre/ Minute
nm	-	Nano meter
pH	-	Negative Logarithm of Hydrogen ion Concentration
rpm	-	Rotations Per Minute
SD	-	Standard Deviation
SE	-	Standard Error
IP	-	Indian Pharmacopoeia
UV - VIS	-	Ultraviolet - Visible

v/v	-	Volume/ Volume
λ	-	Lambda
cm	-	Centimeter
µg/ ml	-	Microgram Per Millilitre
MON	-	Montelukast Sodium
AMB	-	Ambroxol Hydrochloride
LEVO	-	Levocetirizine Dihydrochloride
HPTLC	-	High Performance Thin Layer Chromatography
HPLC	-	High Performance Liquid Chromatography
DMF	-	Dimethyl Formamide
BP	-	British Pharmacopoeia
CI	-	Confidence Interval
ng	-	Nano gram
ng/ ml	-	Nano gram per Millilitre
µg	-	Micro gram

INTRODUCTION

1. INTRODUCTION

(*Anjaneyulu Y. et al, 2006)*

Analytical chemistry deals with quantitative analysis of composition of substances and complex materials in various matrices by measuring at physical and chemical properties of a distinctive constuent of the component or components. “A scientific discipline that develops and applies methods, instruments and strategies to obtain information on the composition and nature of matter in space and time”. Analytical chemistry that assume to be the supporting role of an in spreadable tool in advancing in depth knowledge in any a scientific field. A thorough back ground in analytical chemistry plays a vital role for a chemist in the following ways.

1. To develop and evaluate new procedures.

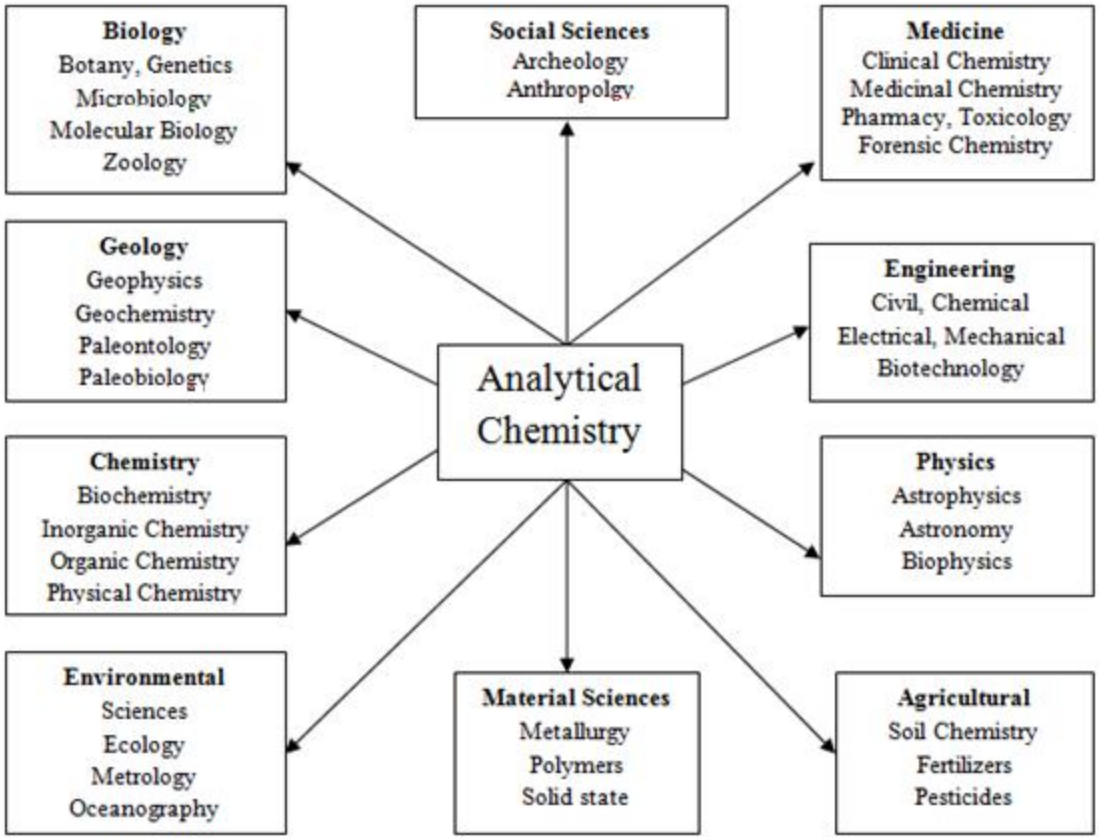
2. Separate simple and complex mixtures.

3. Purity of samples.

4. Write computer programmes statistically to evaluate the reliability of the data.

Modern medicines for human use are required to standards which relate to their quality, safety and efficacy (quantity of the active ingredient). The evaluation of safety and efficacy and their maintenance in practice is dependent upon the existence of adequate methods for quality control of the product. The standard of purity must, therefore, be strictly defined in such a way as to ensure that successive batches are consistent in composition, irrespective of whether they come from the same or different manufactures.

Now a day's analytical chemists are expected to be a vital link an extra ordinary number of diverse fields as follows.



The multi-component formulations have gained a lot of importance now a days due to greater patient acceptability, increased potency and decreased side effects. The quantitative analysis of such multi-component formulations is very important. One of the quantitative procedures for multi-component formulations is the absorbance correction method, which utilizes the measurement of intensity of electromagnetic radiation emitted or absorbed by the analyte. There are various simultaneous estimation methods which are employed for the quantitative estimation of multi-component formulations. The spectrophotometer has become a useful instrument for drug analysis. Now it is the

instrument of choice in conducting quantitative estimation of coloured and colourless solutions.

1.1 ANALYTICAL TECHNIQUES (*Fifeld* F.W *and Kealey D. 2006*)

A wide variety of parameters may be measured by using following techniques.

S.No	Group	Property measured
1	Gravimetric	Weight of pure analyte or of a stoichiometric compound containing it.
2	Volumetric	Volume of standard reagent reacting with the analyte
3	Spectrometric	Intensity of electromagnetic radiation emitted or absorbed by the analyte.
4	Electrochemical	Electrical properties of analyte solutions
5	Radiochemical	Intensity nuclear radiations emitted by the analyte.
6	Mass spectrometric	Abundance of molecular fragments derived from the analyte.
7	Chromatographic	Physico- chemical properties of individual analytes after separation.
8	Thermal	Physico- chemical properties of the sample as it is heated and cooled.

1.1.1 Factors Affecting the Choice of Analytical Method (*Mendham et al., 1994*)

Selection of particular analytical techniques differing in degrees of sophistication, Sensitivity and Selectivity differs in availability, cost and time.

1. Type of chemical analysis required: elemental or molecular, routine or occasional.

2. Problems arising from nature of materials to be investigated.

e.g radioactive substances, corrosive substances and substances affected by water.

3. Possible interferences from components of the material other than those of interest.

4. The concentration range which needs to be investigated.

5. The accuracy required.

6. The time required for complete analysis.

7. The number of analyses of similar type which have to be performed.

1.1.2 The Typical Separation Procedures Include (*Koh Hl., et al., 2003; Jan N., et al., 2011; Segura 2009; Yvan Gaillard 2000 and Mario Theyis et al., 2007*)

Traditionally pharmaceutical analysis referred to chemical analysis of drug molecules, in various combination techniques like chemometrics, micro dosing studies and nano technology. The analytical advances plays its role in drug discovery, analysis of natural products and nutraceuticals, analysis of systemic biology (proteomics, metabolimics and glycomics), biosensors and bioreactors, advances in chiral separations, drug binding analysis, forensic and anti-doping analysis, high sensitivity technologies for trace analysis (Micro and nano scale level), new trends in bio analysis (from urine, hair analysis and exhaled air), Therapeutic drug monitoring and toxicological analysis.

a. Selectivity precipitation of interferants.
b. Masking of specific interferants by complexing agent.
c. Selective oxidation or reduction of interferants.
d. Solvent extraction by converting to suitable form.
e. Ion exchange.
f. Chromatography

Advanced separating and detecting methods include

1. Gas Chromatography – Mass Spectrometry (GC - MS)
2. Liquid Chromatography – Mass Spectrophotometry (LC-MS)
3. Inductively coupled plasma – Mass Spectrometry (ICP - MS)
4. Large Geometry Secondary Ion Mass Spectrometry (LG - SIMS)
5. Super Critical Fluid Chromatography (SFC)
6. Capillary Zone Electrophoresis (CZE)

Usage of LC-MS and GC-MS in clinical, legal and forensic fields is appreciable in the technique of anti doping laboratories. It is generally used to detect that the athlete has taken any drugs could be easily found out by observing the HCG (Human chorionic gonadotropin) level which is a natural anabolic steroid used for excessive strength for athlete. In postmortem drug analysis, LC-MS was generally used to detect special drugs like Heroin, Cocaine, Anti-depressants, Anti-psychotics and Benzodiazepines if present in unstable or degraded form. ICP-MS is required for the detection of metal analysis clinically, especially in heavy metal poisoning cases. Another easy method of detecting anabolic steroids in anti doping laboratories can be done by powdering the hair, treated with methanol and alkaline digestion with sodium hydroxide for the optimum recovery of the drug and could be easily analyzed by gas chromatography coupled to triple quadrapole mass spectrometry, here nitrogen gas was used as carrier gas for gas chromatography. LG-SIMS is used for the detection speed and sensitivity of nuclear material which is used for identification of source of material. Super critical fluid chromatography (SFC) is particularly suitable for moderately polar compounds or mass

sensitive detection. Capillary zone electrophoresis (CZE) has a promising future in analysis of drugs and in the field of biotechnological analysis.

HPLC and GC are most commonly used chromatographic techniques in analyzing starting materials, intermediates and active pharmaceutical ingredients (API) in research and development. HPTLC is very useful technique in solving various problems and can complement other chromatographic techniques also.

Methodology

In developing a quantitative method for determining an unknown concentration of given species by absorption spectrophotometry, the following steps are followed,

1. To record the spectrum of a solution of known concentration of each component.
2. From the spectra, choose the wavelength for each component based at which absorptive measurements are to be made.
3. Prepare a working curve of each pure component at each of chosen wavelengths. Calibration curve for each component are recorded and if straight curves are obtained, such that it obey Beer's law of absorption and absorptivity values are obtained for such curves.
4. Write equations similar to all wavelengths by using absorptivity values
5. Make sure that the absorbance are additive for each concentration at that particular selected wavelength.
6. Determine the absorbance of mixture in all wavelengths selected for analysis and substitute in the equations obtained.
7. Solve the equations simultaneously for each component's concentrations by using matrix algebra (if needed).

1.2 INTRODUCTION TO UV SPECTROSCOPY

(*B.K Sharma, 2006; Robert D. Braun et al., 2006; Gurudeep R. Chatwal et al, 2008*)

The spectral methods of analysis are used for measuring the amount of electromagnetic radiation (EMR) that is absorbed, emitted or scattered by a sample to perform assay. EMR possess discrete energy particles called 'photons', which travel as a wave. In particular ultra-violet visible absorption usually corresponds to excitation of electrons from ground state to higher energy state, by the application of energy which will be generally in the form of photons (EMR). Absorption and emission of radiant energy is the basis for many methods in analytical chemistry. The interpretation of these data gives information regarding qualitative and quantitative analysis.

Qualitatively, the position of absorption and emission lines or bands which occur in the electromagnetic spectrum serve as an indication of the presence of a specific substance. Quantitatively, the intensities of the same absorption and emission lines or bands for the unknown and standards are measured and the concentration of unknown is determined from these data. Depending on the wavelength of incident radiation and molecule can be excited to different vibrational and rotational level; as the energy different associated for an electron level are small. When the energy involved in the electronic transition is large, the absorption will take place primarily in ultra-violet. If the energy absorbed is greater for some visible wavelengths than for others, the emergent beam will appear coloured. The apparent colour of the solution is always the complement of the colour absorbed.

Ultra violet absorption spectra are attributed to a process in which the outer electron of atoms or molecules absorbs radiant energy and undergoes transitions to higher energy level. There transition are quantitized and depend on electronic structure of the

absorbent. Many molecules absorb ultraviolet or visible light. The absorbance of a solution increases as attenuation of the beam increases. Absorbance is directly proportional to the path length, *b*, and the concentration, *c*, of the absorbing species. Beer's Law states that

$\boldsymbol{A = \varepsilon bc}$, Where, $\boldsymbol{\varepsilon}$ is a constant of proportionality, called the absorptivity.

Different molecules absorb radiation of different wavelengths. An absorption spectrum will show a number of absorption bands corresponding to structural groups within the molecule.

1.2.1 Beer - Lambert's Law

When light is incident upon a homogeneous medium, a part of incident light is reflected, a part is absorbed by the medium and the remainder is allowed to transmit as such.

$$I_0 = I_a + I_t + I_r$$

Where,

I_0 = Incident light

I_a = Absorbed light

I_t = Transmitted light

I_r = Reflected light

Lambert's Law states "when a beam of light is allowed to pass through a transparent medium, the rate of decrease of intensity with the thickness of the medium is directly proportional to the intensity of the light".

$$-\frac{dI}{dt} \propto I$$

$$I_t = I_0\, e^{-kt} \quad \text{---------- (1)}$$

(or)

$$In\frac{I_0}{I_t} = kt$$

Beer's Law states "The intensity of a beam of monochromatic light decreases exponentially with the increase in concentration of the absorbing substance".

$$-\frac{dI}{dc} \propto I$$

$$I_t = I_0\, e^{-k'c} \quad \text{---------- (2)}$$

(or)

$$In\frac{I_0}{I_t} = -k'c$$

By solving equations 1 and 2, on changing equations from natural logarithm,

$$I_t = I_0.\ 10^{-0.4343kt} = I_0\ 10^{-kt} \quad \text{---------- (3)}$$

$$I_t = I_0.\ 10^{-0.4343\ k'c} = I_0^{-k'c} \quad \text{---------- (4)}$$

On combining equations 3 and 4,

$$I_t = I_0\ 10^{-act}$$

$$\log\frac{I_0}{I_t} = act$$

Where k and k' are constants, C is the concentration of the absorbing substance and t denotes thickness of the medium.

1.2.1.1 Limitations of Beer's law

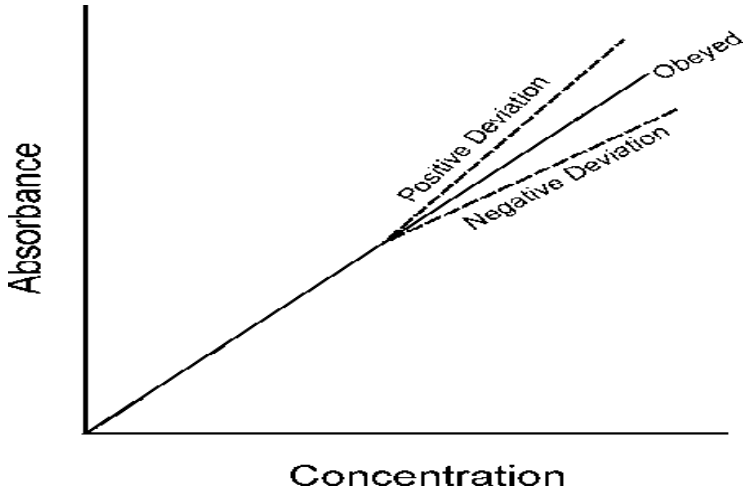
The linearity of the Beer-Lambert law is limited by chemical and instrumental factors. Causes of nonlinearity include:

- deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between molecules in close proximity
- scattering of light due to particulates in the sample
- fluorescence or phosphorescence of the sample
- changes in refractive index at high analyte concentration
- shifts in chemical equilibria as a function of concentration

- non-monochromatic radiation, deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band
- stray light

1.2.1.2 Deviations from Beer's law

According to Beer's law, a straight line passing through the origin should be obtained, when a graph is plotted between absorbance and concentration. But there is always a deviation from linear relationship between absorbance and concentration and intact the shape of an absorption curve usually changes with changes in concentration of solution and unless precautions are observed. Deviations from the law may be positive or negative according to whether the resulting curve is concave upward or concave downward.



The latter two are generally known as instrumental deviation and chemical deviation.

a. Instrumental deviations

Stray radiation, Improper slit width, Fluctuation in single beam.

b. Chemical deviations

Hydrolysis, Association, Polymerization, Ionization and Hydrogen bonding

1.2.1.3 Deviations from Beer's law can arise due to the following factors

1. Beer's law will hold over a wide range of concentration provided the structure of coloured ion or of the coloured non electrolyte in the dissolved state does not change with concentration. If a coloured solution is having a foreign substance whose ions do not react chemically with the coloured components, its small concentration does not affect the light absorption and may also alter the value of extinction co - efficient.
2. Deviations may also occur if the coloured solute ions dissociates or associates.
3. Deviations may also occur due to the presence of impurities that fluorescence or absorb at absorption wavelength.
4. Deviations may occur if monochromatic light is not used.
5. Deviations may occur if the width of slit is not proper and therefore it allows undesirable radiations to fall on the detector.
6. Deviations may occur if the solution undergoes polymerization.
7. Beer's law cannot apply to suspensions but the latter can estimated calorimetrically after preparing a reference curve with known concentrations.

1.2.2 Choice of Solvent *(William Kemp, 2006)*

A suitable solvent for ultraviolet spectroscopy should meet the following requirements.

(i) It should not itself absorb radiations in the region under investigation.

(ii) It should be less polar so that it has minimum interaction with the solute molecule.

1.2.2.1 Solvents used in UV spectroscopy

S.No.	Solvent	Cut-off (nm)
1.	Ethanol	205
2.	Methanol	210
3.	Acetonitrile	210
4.	Hexane	210
5.	Cyclo hexane	210
6.	Diethyl ether	220
7.	Chloroform	245
8.	Carbon tetrachloride	265
9.	Toluene	280
10.	Acetone	330

1.2.3 Electronic Transitions *(B.K Sharma, 2006; Willard et al, 1986; Skoog et al, 2006)*

All organic compounds or compounds are capable of absorbing EMR (Electro Magnetic Radiation) because all containing valance electrons that can be excited to higher energy levels. The electron that contributes to the absorption characteristics of an organic molecule includes,

a) Electrons participating in bond formation between atoms of a molecule

b) Non bonding or unshared outer electrons that are localized like oxygen, sulphur, nitrogen and halogens.

There are three different types of electrons which may be present in an organic molecule include:

a) Sigma (σ) electrons

- Associated with saturated bonds, so called as sigma electrons
- Electrons remains localized in direction of inter nuclear axis
- High energy bonds, so not absorbed in UV region
- Generally used as solvents in UV.

b) Pi (π) electrons

- Associated with unsaturated bonds so called as Pi electrons
- Electrons remain localized in perpendicular to inter nuclear axis
- Moderate energy bonds, which could be excited by UV radiation so, compounds with pi electrons could be detected are examined by UV.

c) Non bonding (n) electrons

- Less firmly held than any another electrons
- Present in atoms like oxygen, nitrogen, sulphur and halogens
- Easily excited by UV radiation.
- Generally forms coordinate covalent bonds

The excited states of any electrons which are involved in bond formation are called as anti-bonding orbitals. As ‘n’ electrons do not form bonds, there are no anti-bonding orbitals associated with them.

1.2.3.1 Important types of electronic transitions with examples are shown below

S.No	Transition type	Energy for excitation	Examples
1.	σ - σ*	Very high	Alkanes – Methane, Ethane.
2.	n - σ*	High	Alcohols, Ethers, Thiols and Disulphides. Alkyl halides – Methyl Iodide, Methyl chloride.
3.	π - π*	Moderate	Alkenes – Ethylene. Alkynes – Ethyne and Propyne. Carbonyl compounds.
4.	n - π*	Low	Carbonyl compounds, Pyridine

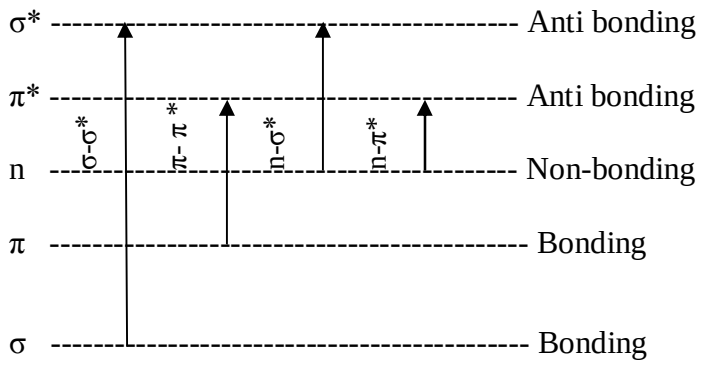


Figure: Electronic Transitions

1.2.4 Instrumentation

(Gurudeep R. Chatwal et al., 2008)
(http://www2.chemistry.msu.edu/faculty/reusch/virtTxtJml/Spectrpy/UVVis/uvspec.html)

All photometers, colorimeters and spectrophotometers have the following basic components

1.2.4.1 Radiation source

i) It must be stable.

ii) It must be of sufficient intensity for the transmitted energy to be detected at the end of the optical path.

iii) It must supply continuous radiation over the entire wavelength region in which it is used.

1.2.4.1.1 *UV region*

Hydrogen discharge lamp, Deuterium discharge lamp, Xenon arc Lamp

1.2.4.1.2 *Visible region*

The tungsten lamp and tungsten halogen lamp are the most common source of visible radiation.

1.2.4.2 Filters and monochromators

The filters and monochromators are used to disperse the radiation according to the wavelength.

1.2.4.2.1 *Filters*

A light filter is a device that allows light of the required wavelength to pass but absorbs light of other wavelengths wholly or partially. Thus, a suitable filter can be selecting a desired wavelength band. It means that a particular filter may be used for a specific analysis. If analysis is carried out for several species, a large number of filters have to be used and interchanged. This method is very useful for routine analysis.

Types of filters

Filters are two types,

i) Absorption filters

ii) Interference filters

Absorption filters work by selective absorption of unwanted wavelengths and are made up of solid sheet of glass, coloured by a pigment or dispersed in glass and dyed gelatin. Interference filters work by selective transmission of selected wavelengths and they are made up of semitransparent metal film deposited on a glass plate and coated with dielectric material (MgF_2).

1.2.4.2.2 *Monochromators*

Monochromators successfully isolates band of wavelengths usually much more than a narrower filter. The essential elements of a monochromator are an entrance slit, a dispersing element (prim or gratings) and an exit slit. The entrance slit sharply defines the incoming beam of heterochromatic radiation. The dispersing element disperses the heterochromatic radiation into its component wavelengths where as exit slit allows the nominal wavelength together with a band of wavelength on either side of it. The position of the dispersing element is always adjusted by rotating it to vary the nominal wavelength passing through the exit slit.

Types of monochromator

1) Prisms

2) Gratings

A prism is made up of quartz (for UV region), glass (for visual range) and alkali halides (for IR). The main advantage of prisms is that they undergo dispersion giving wavelengths which do not overlap, but the main disadvantage is that they give non – linear dispersion. A grating consists of large number of parallel lines ruled on a

highly polished surface like alumina. Generally, gratings are difficult to prepare therefore, replica gratings are prepared from an original grating. This is done by coating the original grating with a film of an epoxy resin which after setting is removed to yield replica. Then replica is made reflective by aluminizing its surface. Gratings give linear dispersion but they suffer from an overlap of spectral orders.

1.2.4.3 Sample cells

These are containers for holding the sample and reference solutions and must be transparent to the radiation passing through generally with a thickness of 1 Cm. The choice of a sample cells are based on transmission characteristics at desired wave lengths, the path length, shape, size and the relative expense. The transmission characteristics are based on the construction materials. For UV region, the cells made up of quartz and for visible region, the cells are made of glass.

1.2.4.4 Detectors

Detectors used in UV-Visible spectrophotometers can be called as photometric detectors. In these detectors the light energy is converted to electrical signal which can be recorded. The types of detectors used are Barrier Layer cell (or) Photo Voltaic cell, Photo tubes (or) Photo emissive tubes, Photomultiplier tubes and Photo diode.

1.2.4.5 Recorders

Detectors transmits the amount of light absorbed by a particular chemical species and only by that species is desired and by correcting the absorbance of solvent and other species in the solution. The recorders record the spectrum without any interferences compared with blank and they are user friendly.

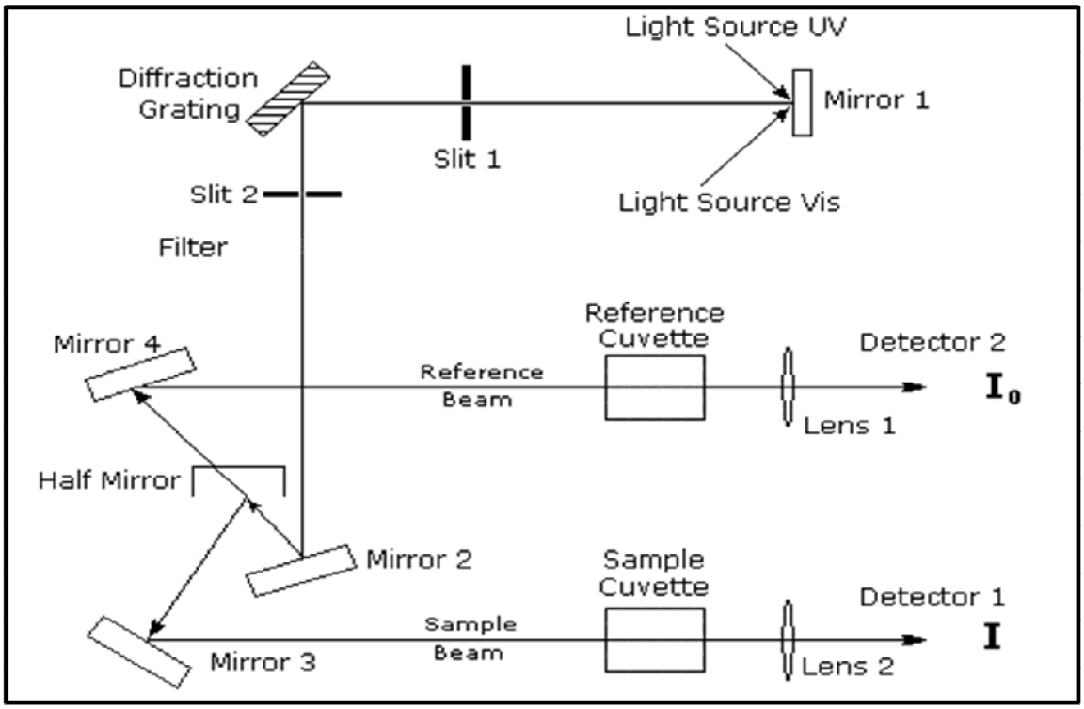


Figure: Double Beam UV - Spectrophotometer

1.2.5 Spectrophotometric Multi - Component Analysis

(*A.H Bekette and Stenlake J.B., 2002*)

Absorption spectroscopy is one of the most useful and widely used tools available to the analyst for quantitative analysis. The relation between the concentration of analyte and the amount of light absorbed is the basis of most analytical applications of molecular spectroscopy. This method of analysis is gaining importance due to simple, rapid, precise, spectra of highly accurate and less time consuming. Spectrophotometric multi-component analysis can be applied where the drugs overlaps. In such cases of overlapping spectra, simultaneous equation can be framed to obtain the concentration of individual component otherwise multi-component analysis can be applied on any degree of spectral overlap provided that two or more spectra are not similar. The various spectroscopic techniques used for multi-component analysis include

1. Simultaneous equation method

2. Absorption ratio method
3. Geometric correction method
4. Absorption correction method
5. Orthogonal polynomial method
6. Differential spectroscopy
7. Derivative spectroscopy
8. Area under curve method.

The various chromatographic techniques helps in multi-component analysis include

1. High performance liquid chromatography
2. High performance thin layer chromatography
3. Gas chromatography

1.2.5.1 Different spectroscopic methods

The assay of an absorbing substance may be quickly carried out by preparing a solution in a transparent solvent and measuring its absorbance at a suitable wavelength. The wavelength normally selected is a wavelength of maximum absorption (λ_{max}), where small errors in setting the wavelength scale have little effects on the measured absorbance.

1.2.5.1.1 ***Assay of substances in single component samples***

Absorption spectroscopy is one of the most useful tools available to the chemist for quantitative analysis. The most important characteristics of photometer and spectrophotometric method are high selectivity and ease of convenience. Quantitative analysis (assay of an absorbing substance) can be done using following methods.

- Use of $A^{1\%}_{1\ cm}$ values

- Use of calibration graph (multiple standard method)
- By single or double point standardization method.

1.2.5.1.1.1 *Use of $A_{1\,cm}^{1\%}$ values*

This method can be used for estimation of drug from formulations or raw material, when reference standard not available. The use of standard value $A_{1\,cm}^{1\%}$ avoids the need to prepare a standard solution of the reference substance in order to determine its absorptivity, and is of advantage in situations where it is difficult or expensive to obtain a sample of the reference substance.

1.2.5.1.1.2 *Use of calibration graph*

In this procedure the absorbances of a number (typically 4 - 6) of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution. Calibration data are essential if the absorbance has a non-linear relationship with concentration, or if the absorbance or linearity is dependent on the assay conditions. In certain visible spectrophotometric assays of colourless substances, based upon conversion to coloured derivatives by heating the substance with one or more reagents, slight variation of assay conditions, e.g. P^H, temperature and time of heating, may rise to a significant variation of absorbance, and experimentally derived calibration data are required for each set of samples.

1.2.5.1.1.3 *Single or double point standardization*

The single point procedure involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The standard and

the sample solution are prepared in similar manner; ideally the concentration of the standard solution should be close to that of the sample solution. The concentration of the substance in the sample is calculated using following formula.

$$C_{test} = A_{test} \times C_{std} / A_{std}$$

Where,

C_{test} and C_{std} are the concentration in the sample and standard solutions respectively.

A_{test} and A_{std} are the absorbance of the sample and standard solutions respectively.

In double point standardization, the concentration of one of the standard solution is greater than that of the sample while the other standard solution has a lower concentration than the sample. The concentration of the substance in the sample solution is given by

$$C_{test} = \frac{(A_{test} - A_{std1})(C_{std1} - C_{std2}) + C_{std1}(A_{std1} - A_{std2})}{A_{std1} - A_{std2}}$$

Where,

C_{std} is the concentration of the standard solution.

A_{test} and A_{std} are the absorbance of the sample and standard solution respectively.

Std_1 and Std_2 are the more concentrated standard and less concentrated standard respectively.

1.2.5.1.2 *Assay of substances in Multicomponent Samples*

The spectrophotometric assay of drugs rarely involves the measurement of absorbance of samples containing only one absorbing component. The pharmaceutical

analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances which potentially interfere in the assay. If the recipe of the sample formulation is available to the analyst, the identity and concentration of the interferents are known and the extent of interference is the assay may be determined. Alternatively, interference which is difficult to quantify may arise in the analysis of formulations from manufacturing impurities, decomposition products and formulation excepients. Unwanted absorption from this source is termed irrelevant absorption and, if not removed, imparts a systematic error to the assay of the drug in the sample.

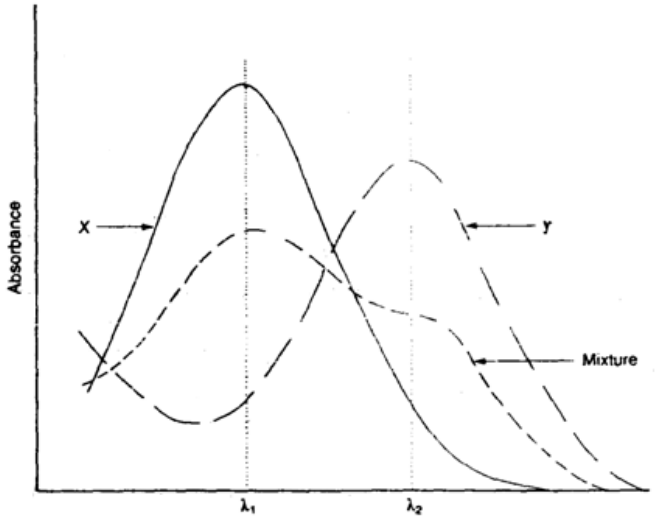
A number of modifications to the simple spectrophotometric procedure described above for single-component samples are available to the analyst, which may eliminate certain sources of interference and permit the accurate determination of one or all of the absorbing components. Each modification of the basic procedure may be applied if certain criteria are satisfied. The correct choice of procedure for a particular analytical problem provides the analyst with a opportunity to demonstrate his/her analytical expertise.

The basic of all the spectrophotometric techniques for multicomponent samples is the property that at all wavelengths:

a. The absorbance of a solution is the sum of absorbance of the individual components, or
b. The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

1.2.5.1.2.1 *Simultaneous equations method*

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λmax of the other, it may be possible to determine both drugs by the technique of simultaneous equations (Vierodt's method)



The information required is:

a. The absorptivities of X at λ_1 and λ_2, a_{x1} and a_{x2} respectively.

b. The absorptivities of Y at λ_1 and λ_2, a_{y1} and a_{y2} respectively.

c. The absorbances of the diluted sample at λ_1 and λ_2, A_1 and A_2 respectively.

Let Cx and Cy be the concentrations of X and Y respectively for diluted sample.

Two equations are constructed based upon the fact that at λ_1 and λ_2 the absorbance of the mixture is the sum of individual absorbances of X and Y.

At λ_1

$$A_1 = ax_1bcx + ay_1bcy \qquad (1)$$

At λ_2

$$A_1 = ax_2bc_x + ay_2bc_y \qquad (2)$$

For measurements in 1 cm cells, b = 1

Rearrange eq. (2)

$$c_y = \frac{A_2 - a_{x2}c_x}{a_{y2}}$$

Substituting for c_y in eq. (1) and rearranging gives

$$C_x = \frac{A_2 a_{y1} - A_1 a_{y2}}{a_{x2}a_{y1} - a_{x1}a_{y2}}$$

And

$$C_y = \frac{A_1 a_{x2} - A_2 a_{x1}}{a_{x2}a_{y1} - a_{x1}a_{y2}}$$

As an exercise you should derive modified equations containing a symbol (b) for path length, for application in situations where A_1 and A_2 are measured in cells other than 1 cm path length.

Criteria for obtaining maximum precision, based upon absorbance ratios, have been suggested that place limits on the relative concentrations of the components of the mixture. The criteria are that the ratios

$$\frac{A_2/A_1}{a_{x2}/a_{x1}} and \frac{a_{y2}/a_{y1}}{A_2/A_1}$$

should lie outside the range 0.1 - 2.0 for the precise determination of Y and X respectively, these criteria are satisfied only when the λ_{max} of the two components are reasonably dissimilar. An additional criterion is that the two components do not interact chemically, thereby negating the initial assumption that the total absorbance is the sum of the individual absorbance. The additivity of the absorbance should always be confirmed in the development of a new application of this technique.

1.2.5.1.2.2 ***Absorbance Ratio Method***

The absorbance ratio method is a modification of the simultaneous equations procedure. It depends on the property that, for a substance which obeys Beer's Law at all wavelengths, the ratio of absorbances at any two wavelengths is a constant value independent of concentration or path length. For example, two different dilutions of the same substance give the same absorbance ratio A_1/A_2, 2.0. In the USP, this ratio is referred to as a Q value. The British Pharmacopoeia also uses a ratio of absorbance at specified wavelengths in certain confirmatory tests of identity.

In the quantitative assay of two components in admixture by the absorbance ratio method, absorbances are measured at two wavelengths one being the λ_{max} of one of the components (λ_2) and the other being a wavelength of equal absorptivity of the two components (λ_1), i.e., an iso-absorptive point. Two equations are constructed as described above for the method of simultaneous equation (eq. (1) and eq. (2)). Their treatment is somewhat different, however, and uses the relationship $a_x = a_{y1}$ at (λ_1).

Assume b = 1 cm.

$$A_1 = a_{x1}c_x + a_{x1}C_Y \qquad (5)$$

$$\frac{A_2}{A_1} = \frac{a_{x2}c_x + a_{y2}c_y}{a_{x1}c_x + a_{x1}c_y}$$

Divide each term by $c_x + c_y$ and let $F_x = c_x/(c_x + c_y)$ and $F_y = c_y/(c_x + c_y)$ i.e. Fx and Fy are the fractions of X and Y respectively in the mixture:

$$\frac{A_2}{A_1} = \frac{a_{x2}F_x + a_{y2}F_y}{A_{x1}F_x + a_{x1}F_y}$$

But $F_y = 1 - F_x$,

$$\frac{A_2}{A_1} = \frac{F_x a_{x2} - F_x a_{y2} + a_{y2}}{a_{x1}}$$

$$\frac{A_2}{A_1} = \frac{F_x a_{x2}}{a_{x1}} - \frac{F_x a_{y2}}{a_{y1}} + \frac{a_{y2}}{a_{y1}}$$

Let $Q_x = \frac{a_{x2}}{a_{x1}}, Q_y \frac{a_{y2}}{a_{y1}} and\ Q_M \frac{A_2}{A_1}$

$$Q_M = F_x(Q_x - Q_y) + Q_y$$

$$F_x = \frac{Q_M - Q_y}{Q_x - Q_y} \qquad (6)$$

Equation 6 gives the fraction, rather than the concentration of X (and consequently of Y) in the mixture in terms of absorbance ratios. As these are independent of concentration, only approximate, rather than accurate, dilutions of X, Y and the sample mixture are required to determine Q_x, Q_y and Q_M respectively.

$$A_1 = a_{x1} + (c_x + c_Y)$$

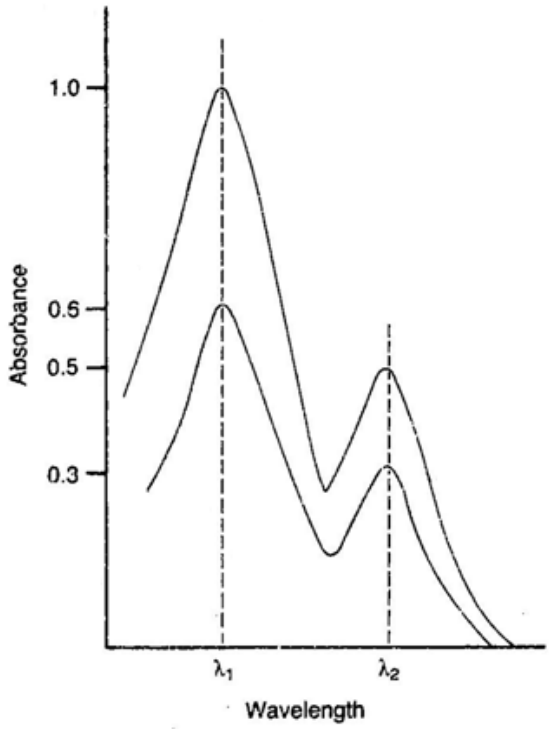
$$c_x + c_y = \frac{A_1}{a_{x1}}$$

From eq. (6)

$$\frac{c_x}{f2c_x + c_y} = \frac{Q_m - Q_y}{Q_x - Q_y b7}$$

$$\frac{c_x}{A_1/a_{x1}} = \frac{Q_M - Q_y}{Q_x - Q_y}$$

$$c_x = \frac{Q_m - Q_y}{Q_x - Q_Y} \cdot \frac{A_1}{a_{x1}} \qquad (7)$$



Equation 7, gives the concentration of X in terms of absorbance ratios, the absorbance of the mixture and the absorptivity of the compounds at the iso-absorptive wavelength. Accurate dilutions of the sample solution and of the standard solutions of X and Y are necessary for the accurate measurement of A_1 and ax_1 respectively.

1.2.5.1.2.3 ***Geometric Correction Method***

A number of mathematical correction procedures have been developed which reduce or eliminate the background irrelevant absorption that may be present in samples of biological origin. The simplest of these procedures is the three-point geometric procedure, which may be applied if the irrelevant absorption is linear at the three wavelengths selected.

$$\text{Corrected absorbance, D} = \frac{y(A2-A3)+ z(A2-A1)}{(Y+Z)(1-r)}$$

1.2.5.1.2.4 *Orthogonal Polynomial Method*

The technique of orthogonal polynomials is another mathematical correction procedure which involves more complex calculations than the three-point correction procedure. The basis of the method is that an absorption spectrum may be represented in terms of orthogonal functions as follows

$$A(\lambda) = p_0P_0(\lambda) + p_1P_1(\lambda) + p_2P_2(\lambda)\ldots.p_nP_n(\lambda)$$

Where, A = Absorbance

λ = Wave length

$P_o(\lambda), P_1(\lambda), P_2(\lambda)\ldots.P_n(\lambda)$ represent the polynomial coefficient

Each coefficient is proportional to each other. These polynomials represent a series of fundamental shapes and the contribution that each shape, e.g.P_2 makes to the absorption spectrum is defined by the appropriate coefficient, e.g. p_2 for P_2. The coefficients are proportional to the concentration of the absorbing analyte, and a modified Beer – Lambert equation may be constructed:

$$p_j = \alpha_j bc$$

For example, when b is 1 cm and concentration of the analyte (c), is in g/ dl. When irrelevant absorption so, present in a sample solution, the calculated coefficient (p_i) comprises the coefficients of the analyte and of the irrelevant absorption (Z).

Thus,

$$P_j = \alpha_j c + p_j(Z)$$

Where,

P_j = polynomial coefficient

a_j = proportionality constant

b = path length

c = concentration

With the correct choice of polynomial, number of wavelengths and the wavelength interval, the contribution from the irrelevant absorption may be negligible. In general, a quadratic (P_2) polynomial eliminates linear or almost linear irrelevant absorption and a cubic (P_3) polynomial eliminates parabolic irrelevant absorption.

The segment of the spectrum of the drug between $\lambda_1$and λ_8 shows a minimum around λ_3 and a maximum around λ_5. Its shape may therefore be represented by a cubic polynomial. The irrelevant absorption is a simple parabolic curve which does not contain a cubic contribution. The coefficient (P_3) of the polynomial for each set of eight absorbances (A_1……..A_8) is calculated from:

$$P_3 = [(-7)\,A_1 + (+5)\,A_2 + (+7)\,A_3 + (+3)\,A_4 + (-3)\,A_5 + (-7)\,A_6 + (-5)\,A_7 + (+7)\,A_8]$$

Where the factors are those of an eight –point cubic polynomial obtained from standard texts of numerical analysis (e.g. Fischer and Yates, 1953). The contribution of the irrelevant absorption to the coefficient of the polynomial of the sample is eliminated by the selection of these parameters, and the concentration of the drug in the sample may be calculate with reference to a standard solution of the drug, from the proportional relationship that exists between the calculated P_3 value and concentration.

The accuracy of the orthogonal functions procedure depends on the correct choice of polynomial order and set of wavelengths. Usually, quadratic or cubic polynomials are selected depending on the shape of the absorption spectra of the drug and the irrelevant absorption. The set of wavelengths is defined by the number of wavelengths, the interval, and the mean wavelength of the set (λmax). approximately linear irrelevant absorption is

normally eliminated using six to eight wavelengths, although many more, up to 20, wavelengths may be required if the irrelevant absorption contains high frequency components. The wavelength interval and λm are best obtained from convoluted absorption curve. This is a plot of the coefficient (P_j) for a specified order of polynomial, a specified number of wavelengths and a specified wavelength interval (on the ordinate) against the λm of the set of wavelengths. The optimum set of wavelengths corresponds with a maximum or minimum in the convoluted curve of the analyte and with a coefficient of zero in the convoluted curve of the irrelevant absorption. In favourable circumstances the concentration of an absorbing drug in admixture with another may be calculated if the correct choice of polynomial parameters is made, thereby eliminating the contribution of one drug from polynomial of the mixture. For, example, the selective assay phenobarbitone, combined with phenytoin in a capsule formulation using a six-point quadratic polynomial, has been reported.

The determination of the optimum set of wavelengths is readily accomplished with the aid of a microcomputer. A suitable exercise is to write a program to compute and plot the data for convoluted spectrum.

1.2.5.1.2.5 ***Difference Spectrophotometry***

The selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferants may be markedly improved by the technique of difference spectrophotometry. The essential feature of a difference spectrophotometric assay is that the measured value is the difference absorbance (ΔA) between two equimolar solutions of the analyte in different chemical forms which exhibit different spectral characteristics.

The criteria for applying difference spectrophotometry to the assay of a substance in the presence of other absorbing substances are that:

a. reproducible changes may be induced in the spectrum of the analyte by the addition of one or more reagents

b. the absorbance of the interfering substances is not altered by the reagents.

The simplest and most commonly employed technique for altering the spectral properties of the analyte is the adjustment of the pH by means of aqueous solutions of acid, alkali or buffers. The ultraviolet-visible absorption spectra of many substances containing ionisable functional groups, e.g. phenols, aromatic carboxylic acids and amines, are dependent on the state of ionization of the functional groups and consequently on the pH of the solution.

The absorption spectra of equimolar solutions of Phenylephrine, a phenolic sympathomimetic agent, in both 0.1M hydrochloric acid (pH 1) and 0.1M sodium hydroxide (pH 13) are shown in figure. The ionization of the phenolic group in alkaline solution generates an additional n (non-bonded) electron that interacts with the with the ring π electrons to produce a bathochromic shift of the λ_{max} from 271nm in acidic solution to 291 nm and an increase in absorbance at the λ_{max} (hyperchromic effect). The difference absorption spectrum is a plot of the difference in absorbance between the solution at pH 13 and that at pH 1 against wavelength. It may be generated automatically using a double-beam recording spectrophotometer with the solution at pH 13 in the sample cell and the solution at pH 1 in the reference cell. At 257 and 278 nm both solutions have identical absorbance and consequently exhibit zero difference absorbance. Such wavelengths of equal absorptivity of the two species are called isobestic

or iso-absorptive points. Above 278 nm the alkaline solution absorbs more intensely than the acidic solution and the ΔA is therefore positive. Between 257 and 278 nm it has a negative value. The measure value in a quantitative difference spectrophotometric assay is the ΔA at any suitable wavelength measured to the baseline, e.g. ΔA_1 at λ_1 or amplitude between an adjacent maximum and minimum, e.g. ΔA_1 at λ_2 and λ_1.

$$\text{At } \lambda_1 \ \Delta A = A_{alk} - A_{acid}$$

Where A_{alk} and A_{acid} are the individual absorbances in 0.1M sodium hydroxide and 0.1M hydrochloric acid solution respectively. If the individual absorbance, and are proportional to the concentration of the analyte and path length, the also obeys the Beer – Lambert's law and a modified equation may be derived.

Where ΔA is the difference absorptivity of the substance at the wavelength of measurement.

If one or more other absorbing substances are present in the sample which at the analytical wavelength has identical absorbance in the alkaline and acidic solutions, its interference in the spectrophotometric measurement is eliminated. The selectivity of the ΔA procedure depends on the correct choice of the pH values to induce the spectral change of the analyte without altering the absorbance of the interfering components of the sample. The use of 0.1M sodium hydrochloric acid to induce the ΔA of the analyte is convenient and satisfactory when the irrelevant absorption arises from pH intensive substances.

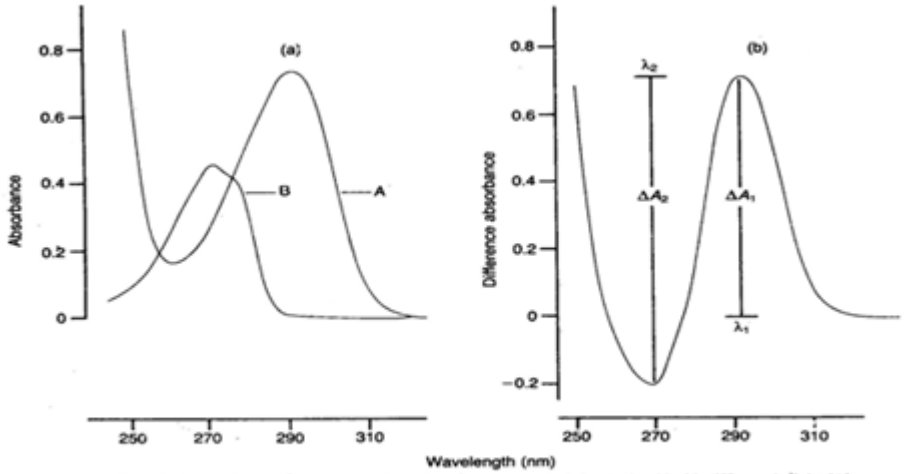


Fig. (a) The absorption spectra of equimolar solutions of phenylephrine hydrochloride (50 μg ml^{-1}) in (A) 0.1M sodium hydroxide and (B) 0.1M hydrochloric acid. (b) The difference absorption spectrum of solution B relative to solution A.

1.2.5.1.2.6 ***Area under the curve method***

From the spectra obtained for calculating the simultaneous equation, the area under the curve were selected at a particular wavelength range for both the drugs were each drug have its absorption. The "X" values of the drugs were determined at the selected AUC range. The "X" value is the ratio of area under the curve at the selected wavelength range with the concentration of the component in mg/ml. These "X" values were the mean of six independent determinations. A set of two simultaneous equations were obtained by using mean "X" values. And further calculations are carried out to obtain the concentration of each drug present in the sample.

1.2.5.1.2.7 ***Absorbance correction method***

The method can be used to calculate the concentration of component of interest found in a mixture containing it along some unwanted interfering component. The absorption different between two points on the mixture spectra is directly proportional to the concentration of the component to be determined irrespective of the interfering component. If the identity, concentration and absorptivity of the absorbing interferences

are known, it is possible to calculate their contribution to the total absorbance of a mixture. The concentration of the absorbing component of interest is then calculated from the corrected absorbance (total absorbance minus the absorbance of the interfering substance) in a usual way. The data required for the construction of absorbance corrected for interference are

i. The λ_{max} of the drugs should be found out by using reference standards of the drugs.

ii. The calibration curve is plotted for each drug and linearity range should be found out.

iii. At one wavelength, one of the drugs shows no absorbance. Hence the other drug was calculated without any interference.

iv. The absorbance values of every drug at the two wave lengths should be measured and the absorptivity values should be calculated.

v. In another wavelength, the absorbance corrected for another drug and the first drug was determined.

1.2.5.1.2.8 ***Derivative spectrophotometry***

Derivative spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum. The transformations that occur in the derivative spectra are understood by reference to a Gaussian band which represents an ideal absorption band. In the context of derivative spectrophotometry, the normal absorption spectrum is referred to as the fundamental, Zeroth order or D° spectrum.

The first derivative (D^1) spectrum is a plot of the ratio of change of absorbance with wavelength against wavelength, i.e. a plot of the slope of the fundamental spectrum

against wavelength or a plot of $dA/d\lambda$ Vs λ. At λ_2 and λ_4, the maximum positive and maximum negative slope respectively in the D°. Spectrums correspond with maximum and a minimum respectively in the D^1 spectrum. The λ_{max} at λ_3 is a wavelength of zero slope and gives $dA/d\lambda = 0$, i.e. a cross-over point, in the D^1Spectrum.

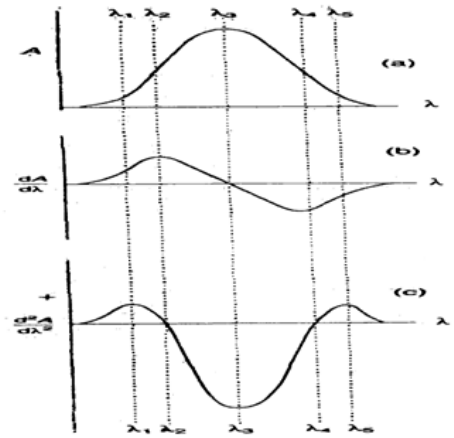


Figure: The zeroth (a), first (b) and second (c) derivative spectra of a Gaussian band

The second derivative (D^2) spectrum is a plot of the curvature of the D° spectrum

against wavelength or a plot of $d^2A/d\lambda^2$ Vs λ. The maximum negative curvature at λ_3 in the D° spectrum gives a minimum in the D^2 spectrum, and at λ_1 and λ_5 the maximum positive curvature in the D° spectrum gives two small maxima called 'satellite' bands in the D^2 spectrum. At λ_2 and λ_4 the wavelengths of maximum slope and zero curvature in the D° spectrum correspond with cross-over points in the D^2 spectrum.

In summary, the first derivative spectrum of an absorption band is characterized by a maximum, a minimum, and a cross-over point at the λ_{max} of the absorption band. The-second derivative spectrum is characterized by two satellite maxima and an inverted band of which the minimum corresponds to the λ_{max} of the fundamental band. As an

exercise, you should construct third and fourth derivative spectra (i.e. plots of $d^3A/d\lambda^3$ and $d^4A/d\lambda^4$ respectively against wavelength) of the fundamental spectrum.

These spectral transformations confer two principal advantages on derivative spectrophotometry. Firstly, an even order spectrum is of narrower spectral bandwidth than its fundamental spectrum. A derivative spectrum therefore shows better resolution of overlapping bands than the fundamental spectrum and may permit the accurate determination of the λ_{max} of the individual bands. Secondly, derivative spectrophotometry discriminates in favors of substances of narrow spectral bandwidth against broad bandwidth substances. This is because 'the derivative amplitude (D), i.e. the distance from a maximum to a minimum, is inversely proportional to the fundamental spectral bandwidth (14') raised to the power (n) of the derivative order.

Thus, $$D \propto (1/W)^n$$

Consequently, substances of narrow spectral bandwidth display larger derivative amplitudes than those of broad bandwidth substances.

(a) The individual spectra of two components X and Y in admixture and their combined spectrum (b) The second derivative spectrum of the mixture showing improved resolution of the individual bands.

These advantages of derivative spectrophotometry, enhanced resolution and bandwidth discrimination, permit the selective determination of certain absorbing substances in samples in which non-specific interference may prohibit the application of simple Spectrophotometric methods. For example, benzenoid drugs such as Ephedrine Hydrochloride, displaying fine structure of narrow spectral bandwidth in the region 240 - 270 nm, are both weakly absorbing (A about 15) and formulated at a relatively low

dose in solid dosage preparations (typically 1 - 50 mg/ unit dose). The high excepients/drug ratio and high sample weight required for the assay may introduce into simple Spectrophotometric procedures serious irrelevant absorption from the formulation excepients. Second derivative spectrophotometry discriminates in favour of the narrow bands of the fine structure of the benzenoid drugs and eliminates the broad band absorption of the excepients. All the amplitudes in the derivative spectrum are proportional to the concentration of the analyte, provided that Beer's Law is obeyed by the fundamental spectrum. The measured value in a quantitative assay is the largest amplitude that is unaffected by the presence of other, absorbing components of the sample spectrophotometric methods. The enhanced resolution and bandwidth discrimination, increases with increasing derivative order. However, it is also found that the concomitant increase in electronic noise. Inherent in the generation of the higher order spectra, the consequent reduction of the signal-to-noise ratio, place serious practical limitations on the higher order spectra.

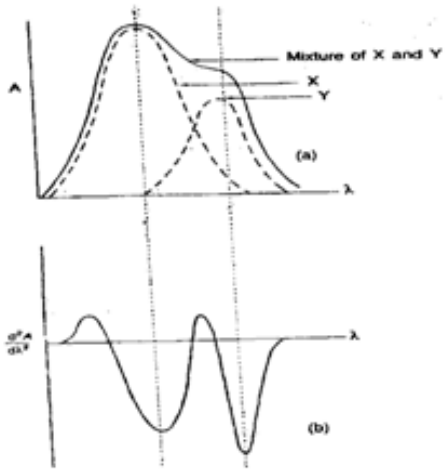


Figure: (a) The individual spectra of two components X and Y in admixture and their combined spectrum (b) The second derivative spectrum of the mixture showing improved resolution of the individual bands.

1.2.6 Different Chromatographic Techniques

1.2.6.1 High performance liquid chromatography (HPLC)

This technique is based on the same method of separation as classical column chromatography, which works on the principle of adsorption, partition, ion exchange or gel permeation, but it differ from column chromatography, as mobile phase is pumped through the packed column under high pressure. The technique is most widely used for all the analytical separation due to its sensitivity, its ready adaptability to accumulate quantitative determinations and its suitability for separating nonvolatile species or thermally fragile ones.

In normal phase HPLC, polar compounds such as silica gel, alumina (Al_2O_3) or porous glass beads were used as column packing materials in stationary phase and non - polar solvents such as heptane, octane or chloroform are used as solvents in mobile phase. In case of reverse phase HPLC, non –polar columns like octa decyl silane (ODS) and C_8 columns are used as stationary phase with polar solvents like water, acetonitrile and methanol are used as mobile phase.

1.2.6.2 High performance thin layer chromatography (HPTLC)

The principle is based on planar chromatography. The mobile phase normally is driven by capillary action. The prominent advantages of this technique includes possibilities of separating of up to 70 samples and standard simultaneously on a single plate leading to high accuracy, low cost and the ability to construct calibration curves from standard chromatography under the same condition as the sample.

1.2.6.3 Gas chromatography (GC)

GC is one of the most extensively used separation technique in which separation is accomplished by partitioning solute between a mobile gas phase and stationary phase. The chief requirement is same degrees of stability at the temperature is necessary to maintain the substance in gas state. Here a carrier gas is used to carry the substance to the column which is having specific characteristics.

1.3 INTRODUCTION FOR HPTLC *(P.D sethi 1996)*

HPTLC is the most simple separation technique today available to the analyst. It can be considered a time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques.

1.3.1 Steps Involved in HPTLC

1. Selection of HPTLC plates and sorbent
2. Sample preparation including any clean up and pre – chromatographic derivatization
3. Application of sample
4. Development (separation)
5. Detection including post – chromatographic derivatization
6. Quantitation
7. Documentation

1.3.2. Plates

1.3.2.1. Precoated plates

The pre – coated plates with different support material (Glass, Aluminum and plastic) and with different sorbent layers are available in the different format and

thickness by various manufacturers. Usually plates with sorbent thickness of 100 – 250 μm are used for qualitative and quantitative analysis, however for preparative TLC work, plates with sorbent thickness of 1.0 – 2.0 mm are available in addition to chemically modified layers. Aluminum Sheet **(**0.1 mm thick): Aluminum sheet as a support offer the same advantage as polyester support but with increased temperature resistance. However with eluents containing high concentration of mineral acids or concentrated ammonia, one may find problem as they will chemically attack aluminum. Aluminum sheets are otherwise compatible with organic solvents and organic acids such as formic acid and acetic acid. Aluminum Precoated plates in size of 20 x 20 cm are usually procured for economic reasons. These plates can be cut to size and shape (format) to suit particular analysis by using general purpose scissors.

1.3.3 Prewashing of Precoated Plates

Sorbents with large surface area absorb not only water vapours and other impurities from atmosphere but other volatile substances often condense particularly after the packing has been opened and exposed to laboratory atmosphere for a long time. Such impurities including elutable components of the binder usually give dirty zones and fail to give reproducible results. It is only for these reasons that pre-coated plates are always packed with the glass or foil side upward (coated layer downward). To avoid any possible interference due to impurities with the chromatographic separations particularly in case of quantitative work, it is always recommended to clear the plates before actual chromatography. This process is called pre washing of plates. Excellent results are obtained if the plates are subjected to pre washing (in cleaning solvent) by continuous mode for some time in a chamber closed by a lid having a slit. After washing, the plates

must be dried for a sufficient time to ensure complete removal of the washing liquids (usually for methanol 30 – 60 minutes at 105° C is required). The washed plates should always be stored in a dust free atmosphere under ambient conditions. As a result of pre washing, signal to noise ratio is substantially low and base lines are straighter, which is essential for quantitative analysis by in situ densitometry. Plates exposed to high humidity or kept on a hand for long time may have to be activated by placing in oven at 110 – 120° C for 30 minutes prior to sample spotting. This step removes water that has been physically absorbed on the surface of the sorbent.

1.3.4 Sample Preparation and Application

The sample preparation procedure is to dissolve the dosage form with complete recovery of intact compounds(s) of interest and minimum of matrix with a suitable concentration of analyte(s) for direct application on the HPTLC plate. Sample application is the most critical step for obtaining good resolution for quantification by HPTLC. The sample should be completely transferred to the layer, however, under no circumstances, the application process should damage the layer, as damaged layer results in unevenly shaped spots. Wherever possible, use of automatic application devices is recommended for quantitative analysis. Usually 0.5 – 5 μl for HPTLC is recommended keeping the size of starting zone(s) down to minimum of 0.5 – 1 mm in concentration range of 0.1 – 1 μg/ ml. Substance zones which are too large from the beginning because poor separation as during development spots does tend to become large and more diffused. It is therefore recommended that solution should be applied in small increments with intermediate drying (use cold or hot air or nitrogen in case of labile compounds, asymmetric accelerated evaporation of the solvent from the point of application can lead to local

changes in the concentration in spotted substances) particularly when the sample solution is predominantly aqueous.

1.3.5 Development (separation)

Poor grade of solvent used in preparing mobile phases have been found to decrease resolution, spot definition and Rf reproducibility. Mobile phases commonly called solvent system is traditionally selected by controlled process of trial and error and also based on one's own experience in the field. Use of mobile phase containing more than three or four components should normally be avoided as it is often difficult to get reproducible ratios of different components. The chamber saturation has pronounced influence on the separation profile. When the plate is introduced in to an unsaturated chamber, during the course of development, the solvent evaporates from the plate mainly at the solvent front. Therefore larger quantity of the solvents shall be required for a given distance; hence resulting is increase in Rf values. If the tank is saturated (by lining with filter paper) prior to development, solvent vapors soon get uniformly distributed throughout the chamber as soon as the plate is placed in such a saturated chamber, it soon gets pre loaded with solvent vapors, hence less solvent shall be required to travel a particular distance, resulting in lower Rf values. Time required for saturation will depend on the nature and composition of mobile phase and layer thickness.

Development in a non-saturated or partially saturated atmosphere is recommended with solvents used in a composition leading to phase separation such as mixture of n - butanol, water and glacial acetic acid. However in case of RPTLC, it is always preferable to saturate the chamber with methanol as normally in RPTLC, mobile phase with high water contents are employed. If chromatographic procedure is to be

carried out at a controlled relative humidity of the chamber then suitable liquid is placed in one of the troughs of twin-trough chamber. Usually relative humidity of the room is controlled by dehumidifier. However, if experiments are required to be carried at specific relative humidity, then solution of sulphuric acid or salt solutions may be employed. After development the plate is removed from the chamber and mobile phase is removed as completely and as quickly as possible. These steps should preferably be performed in fume cup board to avoid contamination of laboratory atmosphere. The plates should always be laid horizontally so that while mobile phase evaporates the separated substances will migrate evenly to the surface where they can be easily detected. There are different factors influencing separation of components and resolution of spots are type of stationary phase – sorbent, particle size, activity, type of plates, layer thickness, pH of layer, binder for layer, melting point, solvent purity, size of chamber, saturation of chamber, solvent for sample phase, melting point level in chamber, size of spot, relative humidity, temperature, flow rate of solvent, separation distant and mode of development.

1.3.6. Detection and visualization

One of the most characteristic features of HPTLC is the possibility to utilize post chromatographic off-line derivatization. With the availability of many visualization reagents, findings can be confirmed. These visualization reactions are possible for identification even if the separation is not optimally. As soon as the development process is complete, the plate is removed from the chamber and dried to remove the mobile phase completely. The zones can be located by various physical, chemical, biological - physiological methods. There is apparently no difficulty in detecting color substances or colorless substances absorbing in short wave UV - region (254 nm) or with

intrinsic fluorescence such as riboflavin. The substance which do not have above properties has to be transferred in to detectable substances by means of chromogenic or fluorogenic reagents which are more expensive, time - consuming and complicated. Detection sensitivity depends on the specificity for the reagent employed. Iodine is the universal detection reagent, that detection is usually non-destructive and reversible but certain substances may be altered through non-reversible derivatization such as ethambutol, a totally non UV absorbing compound forms a UV absorbing complex with iodine through charge transfer. Detection under UV light is the first choice and is non-destructive in most of the cases and is commonly employed for densitometric scanning. Derivatization reactions are essentially required for detection when individual compound does not respond to UV or does not have intrinsic fluorescence. It is not significant whether derivatization is pre or post chromatographic however, pre-chromatographic derivatization not only helps in detection but enhances the selectivity of the mobile phase. For post chromatographic derivatization, smaller the chromatographic zone, greater the concentration of the substance leading to increase in detection sensitivity. Other simple detection method is based on wetting and solubility phenomenon. Aluminium oxide, kieselguhr, silica gel or hydrophilic adsorbents. On dipping or spraying the chromatogram with water, lipophillic substances such as steroids, fatty acids, hydrocarbons appear as white (opaque) spot against semi-transparent back ground as such a substances being invisible with water or not wetted. This wetting effect is more prominent if the plate is fully saturated with water and held against light. The contrast is best immediately after dipping, disappears on drying. Other commonly used reagents are phosphomolybdic acid, antimony trichloride or pentachloride, anisaldehyde - sulphuric

acid, dimethyl amino benzaldehyde in sulphuric acid and fluorescein sodium. These reagents yield sufficient stable coloured spots for quantitative scanning.

1.3.7 Quantitation (Evaluation)

Requirements for various steps in HPTLC are more stringent for quantitative analysis. Accurate and precise application of samples is the most critical. Further, the chromatographic development should clearly and completely separate all the compounds of interest with no loss by decomposition, evaporation or irreversible adsorption during application or development. Sample and standard as a rule should as a rule should be chromatographed on the same plate under similar conditions.

Earlier, a typical approach was scrapping the separated analyte zones from support material and extracting with a suitable solvent, compounds thus eluted could be analysed by any convenient analytical method; spectrophotometric, fluorometric or by suitable colour development method. To compensate for interference from the sorbent, usually a blank area of the layer is also eluted simultaneously and used as a blank for final analysis. Such blank's values can be lowered by pre-washing of TLC layer with methanol, methanol-chloroform (1:1) or methylene chloride prior to chromatographic procedure. However, this method of separating and elution has limited application as the compounds under analysis may be irreversibly bound to the HPTLC supporter elution/isolation steps may cause some chemical transformation or there is likelihood of analyte loss during extraction. Layers containing gypsum as binder are considerably softer and especially suited for preparative chromatography involving scrapping form plate and subsequent elution and estimation.

1.3.7.1 In situ densitometry

Densitometry is the in situ instrumental measurement of visible, UV absorbance, fluorescence or fluorescence quenching directly on the layer without resorting to scrapping and elution. Since chromatographic zones remit a lower light intensity than the environment around it, absorption spectra can be determined directly on the plate by comparison with substance free area on the sorbent layer. The measurements are usually made by reflection from the plate using single beam, double beam or single beam dual wavelength operation of scanning instruments. The purpose of the scanner is to convert the spot or band on the layer into chromatogram. The position of the scanned peaks on the recorder chart are related to Rf values of the spots on the layer and peak height or area is related to the concentration of the substance on the spot. The signals which are measured represent the adsorption of transmitted or reflected light that passes through the spot compared to blank portion of the sorbent layer. A calibration curve consisting of scan area of standard versus amount of analyte is constructed and amount of analyte in the sample represented by scan area is interpolated from the standard curve.

1.3.7.2 Factors influencing in selection of detection wavelength

1. The absorption spectra of compound when recorded in solution or in situ from TLC plate are almost similar. Pre – recorded spectra of these compounds as available in literature may be considered for taking decision.

2. In situ spectra of each component of the formulation may be simultaneously considered for selecting the most suitable wavelength for scanning.

3. Extinction coefficient ($E_{1\,cm}^{1\%}$) and actual concentration of a compound in the formulation has to be taken into consideration, particularly in respect of compounds with low extinction coefficient.

4. While selecting single wavelength, the interest of minor component in the formulation shall need special consideration.

5. If absorption maxima of individual component of the formulation are quite apart, then the chromatogram must be scanned at individual absorption maxima for obtaining meaningful results.

1.3.8 Documentation

The use of application scheme and labeling every single chromatogram can avoid mistake in respect of order of application. It is preferable to apply each sample and reference solution twice by following data pair method. A lead pencil can be used to write on the chromatoplate. The plate should never be marked below the starting point, as the layer is likely to get damaged affecting chromatographic distribution of the substances under analysis, which may ultimately lead to error in scanning. The best way to label the chromatoplate is to mark above the level of solvent point. Immediately after development is completed, the solvent point should be marked with both on left and right hand edges of the plate. To assist the analysts and researchers in practice of HPTLC, E. Merck has recently introduced HPTLC pre coated plates with an imprinted identification code. The data needed for traceability according to HPTLC such as supplier's name, item number, batch number and individual plate number are imprinted near upper edge of the pre coated plates. This will not only help in the traceability of

analytical data, but will avoid manipulation of data at any stage as coding will automatically get recorded during the photo documentation.

1.3.9 Stabilization of developed zones

After treatment with the reagent as part of chromatographic derivatization, coloured or fluorescent chromatographic zones are used for quantitative evaluation. It is therefore desirable that the colour or fluorescence thus produced should be stable at least for 30 minutes for carrying out various steps involved in quantitative analysis. There is no general procedure laid down to stabilize the coloured chromatographic zones except to store in atmosphere of nitrogen and protected from light till they are evaluated. However, fluorescent chromatographs can not only be stabilized but often intensified

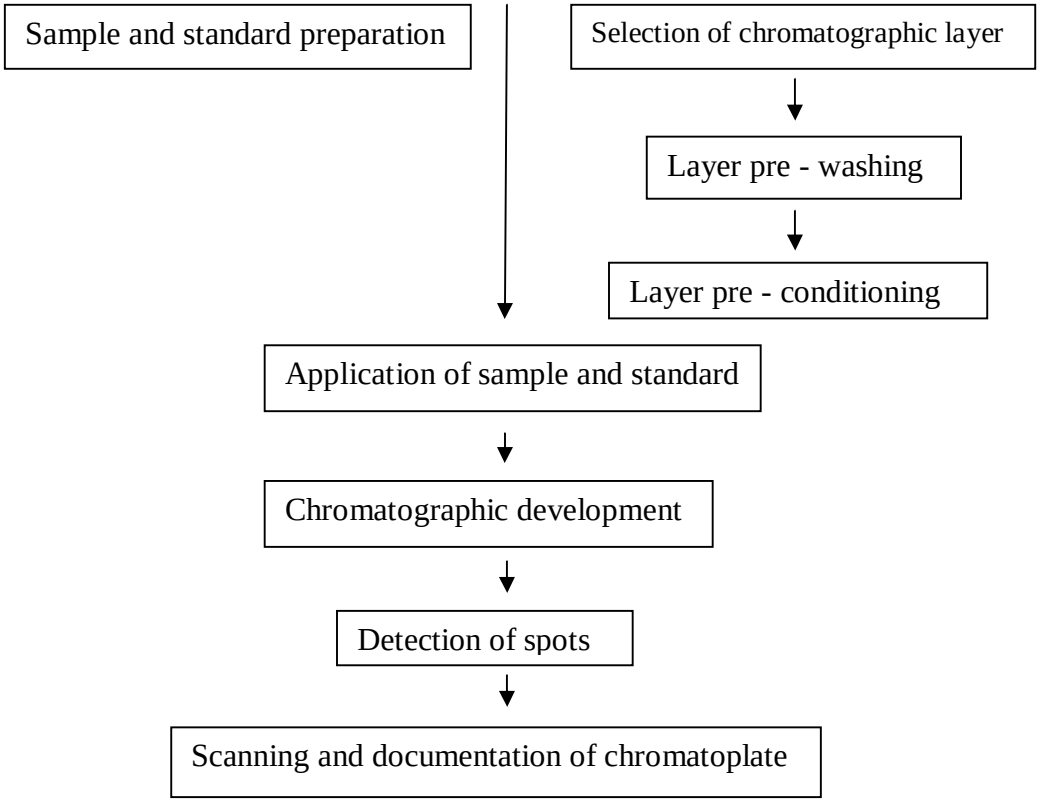


Figure: A detailed layout of HPTLC method development

1.4 ANALYTICAL PARAMETERS USED IN ASSAY VALIDATION AS PER ICH GUIDELINES *(Code Q2A; Q2B, ICH Guidelines 1994 and 1996)*

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included.

Types of Analytical Procedures to be validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures

Identification Tests

- Quantitative tests for impurities content.
- Limit tests for the control of impurities.
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

A brief description of the types of tests considered in this document is provided below.

Identification tests are intended to ensure the identity of an analyte in a sample this is normally achieved by comparison of a property of the sample (example spectrum, chromatographic behaviour, chemical reactivity etc.) to that of a reference standard.

Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than for a limit test.

Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated.

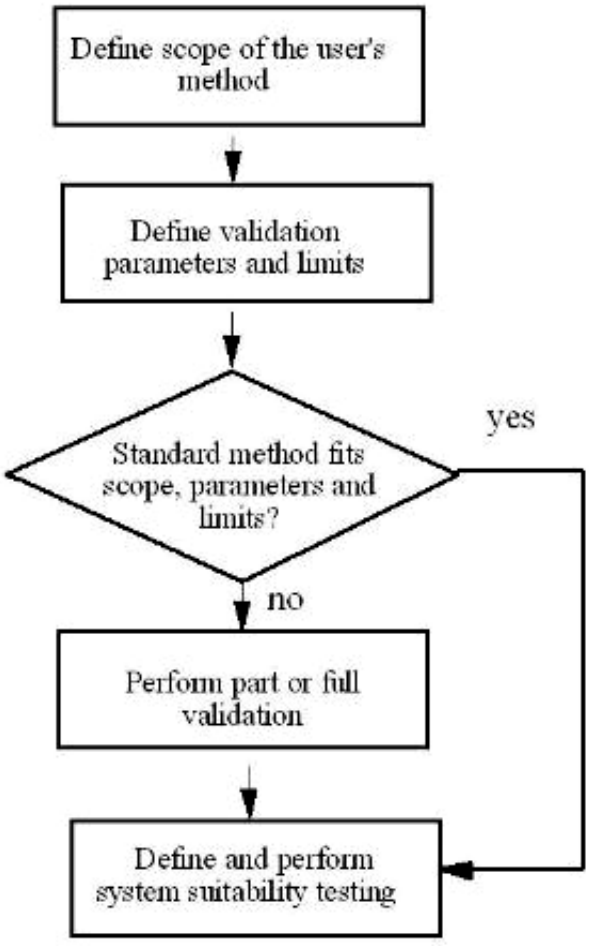


Figure: **Workflow for Evaluation and Validation of Standard Methods**

1.4.1. Typical Validation Parameters

- ❖ Accuracy
- ❖ Precision
- ❖ Range
- ❖ Specificity
- ❖ Linearity
- ❖ Detection Limit
- ❖ Quantification Limit
- ❖ Ruggedness
- ❖ Robustness

1.4.1.1 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or on an accepted reference value and the value found.

1.4.1.2 Precision

It expresses as degree of agreement among individual test results when procedure or method is applied to a homogeneous sample, usually expressed as SD or RSD. It is a measure of degree of repeatability or reproducibility under normal conditions. A more comprehensive definition proposed by the ICH divides precision into three types

1. Repeatability.
2. Intermediate Precision.
3. Reproducibility.

1.4.1.3 Range

The range of a method can be defined as the upper and lower concentrations for which the analytical method has adequate accuracy, precision and linearity. The range of concentrations examined will depend on the type of method and its use.

1.4.1.4 Specificity

Ability of the method to measure accurately and specifically the analyte of interest in presence of matrix and other components likely to be present in the sample matrix and impurities, degradation products and other related substances. For this, one may compare the test results of analysis of samples containing other ingredients or impurities or degradation products or related substances or placebo ingredients with those obtained from analysis of sample without these, i.e., the method must allow distinct analytical measurement of analyte of interest and exclusion of all other relevant interferences. If the impurities or degradation products or potential contaminants are not available one can apply a proposed method to the strain and stress (heat, light, humidity) samples. Degree of agreement among results will explain specificity of the method. If the impurities or degradation products are not available, one may carryout additional purity tests by chromatography-HPLC or HPTLC.

1.4.1.5 Linearity

The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. Linearity can be assessed by performing single measurements at several analyte concentrations.

A linearity correlation coefficient above 0.999 is acceptable for most methods, especially for major components in assay methods. The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample.

1.4.1.6 Detection limit

The Detection Limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantities as an exact value. The detection limit (LOD) may be expressed as

$$LOD = \frac{3.3\sigma}{S}$$

Where,

σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

1.4.1.7 Quantification limit

LOQ is defining as the lowest concentration of the substance (analyte) in a sample that can be estimated quantitatively with acceptable precision, accuracy and reliability by a given method under stated experimental conditions. Quantification Limit (LOQ) may be expressed as

$$LOQ = \frac{10\sigma}{S}$$

Where,

σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

1.4.1.8 Ruggedness

It is the measure of the capacity of the analytical method to remain unaffected by small but deliberate variations in procedure. It provides an indication about variability of the method during normal laboratory conditions.

1.4.1.9 Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "A measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". The most important aspect of robustness is to develop methods that allow for expected variations in the separation parameters.

General acceptance limits

S.No.	Characteristics	Acceptance Criteria
1	Accuracy	Assay limit- 98-102% Recovery - 80,100,120%.with a deviation of $\pm$ 2
2	Precision A)Repeatability b)Intermediate precision	 % RSD < 2 % RSD < 2
3	Specificity/ selectivity	No interference
4	Detection Limit	S/N > 2 or 3
5	Quantitation Limit	S/N > 10
6	Linearity	r > 0.999
7	Range	80-120%
8	Stability	>24hr or > 12hr

1.5 PHARMACEUTICAL STATISTICS *(Gupta S.P, 1994; Mendham, 1994)*

Statistical techniques have been widely used in many diverse areas of scientific investigation. Statistical applications have been recognized as crucial to quality control procedure, test, specification and definitions. Principle of modern analytical techniques and skill in their application are necessary attribute of the successful pharmaceutical analyst, thus does not ensure the satisfactory solution of all the problem that may encountered. Some auxiliary knowledge methods those can aid the analyst in designing experiment, collecting data, and interpreting the result.

1.5.1 Linear Regression

Linear regression is a statistical technique that defines the functional relationship between two variables by best-fitting straight line. Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is

$$y = mx + c$$

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$m = \frac{N\sum xy - (\sum x)(\sum y)}{N\sum x^2 - (\sum x)^2} \qquad \text{and} \qquad c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N\sum x^2 - (\sum x)^2}$$

1.5.2 Correlation Coefficient (r)

It is a procedure commonly used to characterize quantitatively the relationship between variable. Correlation is related to linear regression. To establish whether there is a linear relationship between two variables x_1 and y_1, use Pearson's correlation coefficient r.

$$r = \frac{n\sum x_1 y_1 - \sum x_1 y_1}{\{[n\sum {x_1}^2 - (\sum x_1)^2]\,[n\sum {y_1}^2 - (\sum y_1)^2]\}^{1/2}}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a definite linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1 indicate negative correlation values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

1.5.3 Standard Deviation (SD)

It is commonly used in statistics as a measure of precision statistics as a measure of precision and is more meaningful than is the average deviation. It may be thought of as a root-mean-square deviation of values from their average and is expressed mathematically as

$$S = \sqrt{\frac{\sum_{i=1}^{i=n} (x_i - \bar{x})}{N-1}}$$

Where,

S is standard deviation.

If N is large (50 or more) then of course it is immaterial whether the term in the denomination is N -1 or N

Σ = sum

$\bar{x}$ = Mean or arithmetic average.

$x - \bar{x}$ = deviation of a value from the mean.

N = Number of observations.

1.5 4 Percentage Relative Standard Deviation (%R.S.D)

It is also known as coefficient of variation (CV). It is defined as the standard deviation (S.D) expressed as the percentage of mean.

$$\text{CV or \% RSD} = \frac{S.D}{\bar{x}} \times 100$$

Where,

S.D = the standard deviation,

$\bar{x}$ = Mean or arithmetic average.

The variance is defined as S^2 and is more important in statistics than S itself. However, the latter is much more commonly used with chemical data.

1.5.5 Standard Error of Mean (S.E)

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observations. It is mathematically expressed as

$$S.E = \frac{S.D}{\sqrt{n}}$$

Where,

S.D = Standard deviation.

n = number of observation

1.5.6 Confidence Interval (CI)

A confidence interval gives an estimated range of values which is likely to include a unknown population parameter, the estimated range being calculated from a given set of sample data. A confidence interval with a particular confidence level (95% selected by the user) is intended to give the assurance that, if the statistical model is correct then the interval could deliver the true value.

Confidence interval for a normal population,

$$\mathrm{CI} = \bar{Y} \pm \frac{Z_{\alpha/2}\,\sigma}{\sqrt{N}}$$

Where

$\bar{Y}$ = Sample mean

$Z_{\alpha/2}$ = upper $\alpha/2$ critical value of standard normal distribution

N = Size of sample

σ = Standard deviation

LITERATURE REVIEW

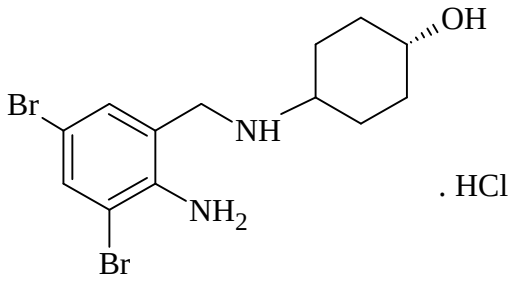
2. LITERATURE REVIEW

2.1. DRUG PROFILE

2.1.1. Ambroxol Hydrochloride

(IP 2007; BP 2009; Martindale, The extra Pharmacopoeia, 2005;The Merck Index 2006; Tripathi K.D, 2003; http://www.en.wikipedia.org/wiki/Ambroxol.html)

Molecular structure



Chemical name

Trans-4-[(2-amino-3, 5-dibromobenzyl) amino] cyclohexanol hydrochloride

Molecular formula

$C_{13}H_{18}Br_2N_2O.HCl$

Molecular weight

414.6 g

Category

Mucolytic expectorant

Description

White or yellowish crystalline powder

Solubility

Ambroxol Hydrochloride is sparignly soluble in water, soluble in methanol, practically insoluble in methylene chloride.

Storage

Store Protected from light.

Identification

i) Melting point

Standard value	Observed average value*
233º C -234.5º C	233.66º C

***Average of six observations**

ii) Infra red spectrum was recorded and shown in figure - 1

Mechanism of action

It is an expectoration improver and mucolytic reagent used in the treatment of acute and chronic disorders characterized by production of excessive thick mucus, which plays an important role in the body's natural defense mechanisms. Ambroxol is a metabolite of bromhexine, which helps in clearance of mucus, facilitates expectoration and eases productive cough. Ambroxol also provides pain relief in acute sore throat by its local anesthetic effect.

Contraindications and Side effects

Generally there are no side effects, but may cause Rhinorrhoea, lacrymation, gastric irritation and Hypersensitivity.

Drug interactions

Administration of Ambroxol together with antibiotics (amoxycilline, cefuroxime, erythromycin and doxycycline) leads to higher antibiotic concentration in the lung tissue.

Pharmacokinetics

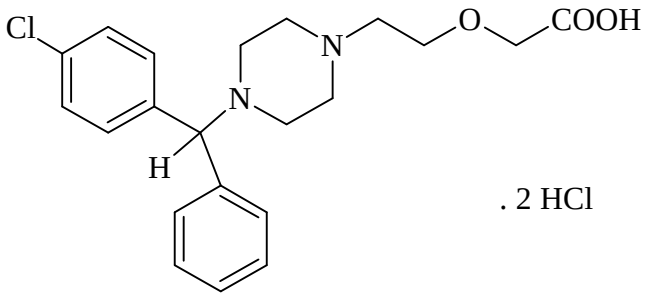
Excretion : Cleared rapidly from the plasma mainly by hepatic metabolism.

Half-life : 4 - 5 minutes (initial); about 40 minutes (terminal).

2.1.2. Levocetirizine Dihydrochloride

(IP 2007; Martindale, The extra Pharmacopoeia, 2005; Goodman and Gillman's 2001; The Merck Index 2006; Clarke's Analysis of Drugs and Poisons 2004; http://www.en.wikipedia.org/wiki/Levocetirizine.html.)

Molecular structure



Chemical name

(R)-2-[2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]aceticacid

dihydrochloride

Molecular formula

$C_{21}H_{25}N_2O_3Cl.2HCl$

Molecular weight

461.8

Category

Anti histamine agent

Description

White or almost white powder

Solubility

It is freely soluble in water, insoluble in acetone and dichloromethane.

Identification

i) Melting point

Standard value	Observed average value*
228 ºC - 229ºC	228.5 ºC

***Average of six observations**

ii) Infra red spectrum was recorded and shown in figure – 2

Storage

Store protected from moisture.

Mechanism of action

It is third-generation non-sedative antihistamine which works by blocking histamine released from mast cells to bind to histaminic receptors, which in turn prevents the release of other allergy chemicals and increased blood supply to that area.

Contraindications and Side effects

General side effects include slight sleepiness, headache, mouth dryness, lightheadedness, blurred vision and fatigue.

Pharmacokinetics

Absorption

Rapidly and extensively absorbed orally and peak plasma concentration attained in 0.9 hour.

Distribution

Apparent volume of distribution is 0.4 L/ kg, expected to distribute in to milk and Plasma protein binding was 91 – 92 % (Mainly Albumin)

Metabolism

Metabolized to a limited extent (< 14 %)

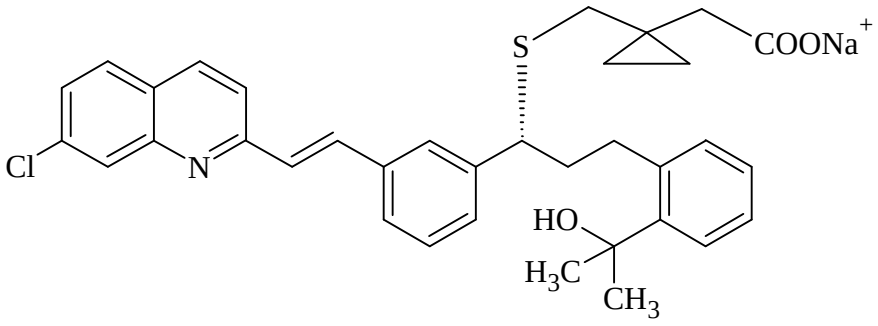
Elimination

Excreted in urine (85.4 %) and in feces (12.9 %)

2.1.3. Montelukast Sodium

(*Goodman and Gillman's, 2001, http://www.drugs.com/mtm/montelukast.html, www.en,wikepedia.org/wiki/Monte.html, Martindale The extra Pharmacopoeia, 2005, The Merck Index, 2006)*

Molecular structure



Chemical name

[R – (E)]-1-[[[1-[3-[2-(7-chloro–2–quinolinyl)ethenyl]phenyl]– 3 – [2-(7-chloro-2-quinolinyl) ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]thio] methyl cyclopropane acetic acid sodium

Molecular Formula

$C_{35}H_{35}ClNNaO_3S$

Molecular weight

608.17

Category

Used in the treatment of Asthma, Allergic Rhinitis and Urticaria.

Description

It is a white to off – white powder.

Solubility

It is freely soluble in methanol, ethanol and water and practically insoluble in acetonitrile

Storage

Store between 59°F to 86°F. Protect from moisture and light.

Identification

i) Melting point

Standard value	**Observed average value***
135.5ºC	133.66ºC

*** Average of six observations**

ii) Infra red spectrum was recorded and shown in figure – 3

Mechanism of action

It binds to cysteinyl leukotriene type 1 ($CysLT_1$) receptor in the upper and lower airways to prevent leukotriene – mediated effects associated with asthma and allergic rhinitis.

Contraindications

Montelukast should not be taken along with Gemfibrozil and prednisolone as the effect of Montelukast (Anti asthmatic) will be enhanced.

Side effects

Consumption of Montelukast may cause cardiac complications, headache, fatigue, abnormal dreams, anxiousness, depression and hallucination.

Pharmacokinetics

Absorption

Oral route of administration and peak plasma concentration was attained within 3 – 4 hours.

Distribution

99 % is bound to plasma and may cross the placenta.

Metabolism

It is extensively metabolized in gastrointestinal tract and liver.

Elimination

It is excreted principally in feces (about 86 %) via bile as unchanged drug.

2.2. REPORTED METHODS

2.2.1. Analytical Methods

2.2.1.1 Ambadas R.Rote *et al.*, (2011) reported "**Determination of Montelukast Sodium and Levocetirizine Dihydrochloride by HPTLC and First Derivative Spectrophotometry".** This method was performed by using precoated silica gel 60 - F_{254} aluminium plate. The mobile phase consists of ethylacetate, methanol and triethanolamine (5:5:0.02 v/v/v) using Paracetamol as internal standard. The detection wavelength was 240 nm. The second method is based on the derivative spectrophotometric method at zero crossing wavelengths of Montelukast Sodium at 291.6 nm and 238.2 nm for Levocetirizine by using methanol as solvent.

2.2.1.2 Arindam Basu *et al.*, (2011) reported "**Simultaneous RP-HPLC Estimation of Levocetirizine hydrochloride and Montelukast Sodium in Tablet Dosage Form".** The method was developed by using Waters HPLC system on a L_7 column (Hypersil Gold: 250 mm x 4.6 mm, 5 μm) using a mixture of methanol and 0.05 M potassium dihydrogen phosphate buffer at pH 7.5 in 80:20 v/v in an isocratic elution mode at a flow rate of 1.2 ml/ min at 35° C and the UV detection was carried out at 225 nm.

2.2.1.3 Patel P.A. *et al.*, (2011) reported "**Spectrophotometric Simultaneous Estimation of Salbutamol and Ambroxol in Bulk and Formulation**" A simultaneous equation method and area under curve method were developed. The solvent used was 0.1 N Hydrochloric acid for both the methods. For simultaneous equation method the wavelength selected were 223 nm and 244 nm. The wavelengths selected for Area Under

Curve method were in the range of 232 - 217 nm for Salbutamol and 252 - 237 nm for Ambroxol.

2.2.1.4 Rahul P.Gunjal *et al*., (2011) reported "**HPLC and LC-MS Studies on Stress Degradation Behavior of Levocetirizine and Development of a Validated Specific Stability – Indicating Method".** The drug was subjected to stress conditions of hydrolysis, photolysis and thermal decomposition and its separation from degradation products was achieved by a C_{18} Supelco column using water and acetonitrile in (50:50 v/v) at a flow rate of 1 ml/ min. Detection was carried out at 230 nm and degraded products were characterized by LC-MS.

2.2.1.5 Sunil R.Dhaneshwar et al., (2011) reported "**Validated HPTLC Method for Simultaneous Estimation of Levocetirizine Hydrochloride and Nimesulide in Formulation**". Chromatographic separation of drugs was performed on silica gel 60 - F_{254} aluminium plates by using a solvent system toluene, ethyl acetate, methanol and ammonia (9:1:1:0.5v/v/v/v) with densitometric evaluation of separated zones at 238 nm.

2.2.1.6 Eswarudu M.M *et al*., (2011) reported "**RP-HPLC Method Development and Validation for Simultaneous Estimation of Montelukast Sodium and Levocetirizine Dihydrochloride in Tablet Dosage Form**". The separation was achieved by using Hypersil C_{18} (250x4.66mm, 5µm) column as a stationary phase and a mixture of acetonitrile and acetate buffer at pH 3.5 (75:25 v/v) at a flow rate of 1 ml/ min with UV detection at 230 nm.

2.2.1.7 Rakshit Kanubhai Trivedi *et al*., (2011) reported "**A Rapid, Stability Indicating RP-UPLC Method for Simultaneous Determination of Ambroxol Hydrochloride, Cetirizine Hydrochloride and Antimicrobial Preservatives in Liquid**

Pharmaceutical Formulation". An Agilent eclipse plus C_{18} (50 x 2.1 mm, 1.8 μm) column was used for the separation with gradient elution technique. The mobile phase used was Acetonitrile and 0.01 M phosphate buffer in 0.1 % triethylamine at a flow rate of 0.5 ml/ min and the detection was carried out at 270 nm.

2.2.1.8 Priyanka A. Patel *et al*., (2011) reported "**Simultaneous Determination of Salbutamol and Ambroxol in Fixed Dose Combination by Spectrophotometry**". An absorbance correction method was developed using 0.1 M Hydrochloric acid. The wavelengths selected were 300 nm for Ambroxol Hydrochloride and 223 nm for Salbutamol and the interference due to Ambroxol was corrected at 223 nm. In first order derivative spectroscopy the wavelengths used for the analysis were 252 nm for Salbutamol and 232 nm for Ambroxol using 0.1 N Hydrochloric acid.

2.2.1.9 Umadevi B. *et al.,* (2011) reported "**Development and Validation of UV Spectrophotometric Determination of Doxofylline and Ambroxol Hydrochloride in Bulk and Combined Tablet Formulation**". A simultaneous equation method and an absorbance correction method were developed. The solvent used was distilled water for both the methods. For simultaneous equation method the wavelength selected were 274 nm and 244.5 nm and for absorbance correction method 308 nm for Ambroxol and 274 nm for Doxofylline and Ambroxol hydrochloride. The interference due to Ambroxol was corrected at 274 nm.

2.2.1.10 Vidhya K.Bhusari *et al.,* (2010) reported "**Application of a Stability-Indicating TLC Method for the Quantitative Determination of Levocetirizine in Pharmaceutical Dosage Forms**". The method employs TLC aluminium plates precoated

with silicagel 60 F_{254} as stationary phase and mobile phase was a mixture of ethyl acetate, methanol and ammonia (9:2.5:1.5 v/v/v) with densitometric detected at 230 nm.

2.2.1.11 Jain P.S. (2010) reported "**Stability-Indicating HPTLC Determination of Ambroxol Hydrochloride in Bulk Drug and Pharmaceutical Dosage Form**". The method employed the usage of pre coated silica gel 60 F_{254} aluminium plates and developed a chromatogram by using methanol and triethylamine (4:6 v/v) and determined densitometrically at 254 nm.

2.2.1.12 Choudhari V. *et al.,* (2010) reported "**Simultaneous Determination of Montelukast Sodium and Levocetirizine Dihydrochloride in Pharmaceutical Preparations by Ratio Derivative Spectroscopy".** The first derivative UV spectroscopy was measured at 250.4 nm for Montelukast Sodium and 238.4 nm for Levocetirizine Dihydrochloride by using methanol as solvent.

2.2.1.13 Shaikh K.A. *et al.,* (2010) reported "**A Stability-Indicating LC Method for the Simultaneous Determination of Levocetirizine Dihydrochloride and Pseudoephedrine Sulfate in Tablet Dosage Forms**". A CosmoSil C_8 (250 x 4.6mm, 5 μm) column with a mobile phase of consists of 0.05M potassium dihydrogen phosphate buffer at pH 3 and 0.25% 1 - octane sulphonic acid sodium salt and acetonitrile were used for separation with gradient elution technique. The flow rate was 1 ml/ min and the detection wavelength was 242 nm using a photo diode array detector.

2.2.1.14 Smitha Sharma *et al.,* (2010) reported "**Development and Validation of TLC-Densitometry Method for Simultaneous Quantification of Montelukast Sodium and Levocetirizine Dihydrochloride Pharmaceutical Solid Dosage Form**". The separation was achieved by using 10 x 10 cm precoated Silicagel 60 - F_{254} aluminium sheets using a

mixture of mobile phase consisting of chloroform, methanol, toluene and glacial acetic acid (10:5:3:0.5v/v/v/v) and developed by using CAMAG TLC Scanner III at 302 nm.

2.2.1.15 Singh R.M. *et al.,* (2010) reported "**Development and Validation of a RP-HPLC Method for Estimation of Montelukast Sodium in Bulk and in Tablet Dosage Form".** The separation was achieved using a Sunfire C_{18} (250 x 4.6 mm, 5 μm) column by using Acetonitrile and 1 mM sodium acetate buffer at pH 6.3 (90:10 v/v) at a flow rate of 1.5 ml/ min and UV detection was carried out at 285 nm.

2.2.1.16 Prasanthi N.L. *et al.,* (2010) reported "**Estimation of Ambroxol Hydrochloride and Guiaphensin in Tablet Dosage Form by Simultaneous Equation Method**". The reported method was simultaneous equation method. The wavelengths selected were 242 nm and 272 nm for the simultaneous estimation Ambroxol and Guiaphensin using methanol as solvent.

2.2.1.17 Ashokkumar. S. *et al*., (2009) reported "**RP-HPLC Method Development and Validation for Simultaneous Estimation of Montelukast Sodium and Levocetirizine Dihydrochloride".** Separation of components were achieved by Phenomenex-Luna, C_{18} column (250 x 4.6 mm, 5 μm) using a mixture of acetonitrile and phosphate buffer at pH 5.5 (35:65 v/v) at flow rate of 1.5 ml/ min. The detection wavelength was 230 nm.

2.2.1.18 Hadad Ghada M. *et al*., (2008) reported "**HPLC and Chemometrics – Assisted UV Spectroscopy Methods for Simultaneous Determination of Ambroxol and Doxocycline in capsule**". Separation was achieved on reverse phase C_{18} column by using a mixture of acetonitrile and 20 mM potassium dihydrogen phosphate buffer at pH 6 (1:1 v/v) and detection was performed at 245 nm.

2.2.1.19 Mirza shahed *et al*., (2008) reported "**Simultaneous Determination of Gatifloxacin and Ambroxol Hydrochloride from Tablet Dosage Form using Reversed - Phase High Performance Liquid Chromatography**". A HiQ sil C_{18} (250 x 4.6 mm, 5 μm) column using dihydrogen ortho phosphate buffer at pH 3 and acetonitrile (70:30 v/v) at flow rate of 1 ml/ min and components were detected by UV detector at 247 nm.

2.2.1.20 Bhatia M. Neela *et al*., (2008) reported "**RP-HPLC and Spectrophotometric Estimation of Ambroxol Hydrochloride and Cetirizine Hydrochloride in Combined Dosage Form**". The chromatographic methods were standardized using a HIQ SIL-C_{18} column (250 x 4.6 mm i.d., 10 μm particle size) with UV detection at 229 nm and mobile phase consisting of methanol, acetonitrile and water (40:40:20 v/v/v). Ambroxol hydrochloride and cetirizine hydrochloride have absorbance maxima at 243 nm and 229 nm, respectively. The iso absorptive wavelength for both the drugs was 236 nm. For absorbance ratio method, the wavelengths selected were 243 nm and 236 nm.

2.2.1.21 Lakshmana Prabhu S. *et al.*, (2008) reported "**Simultaneous UV Spectrophotometric Estimation of Ambroxol hydrochloride and Levocetirizine Dihydrochloride".** The method involved solving simultaneous equations based on measurement of absorbance at 242 nm for Ambroxol Hydrochloride and at 231 nm for Levocetirizine Dihydrochloride by using distilled water as solvent.

2.2.1.22 Al Omari Mahmoud M. *et al*., (2007) reported "**Effect of Light and Heat on the Stability of Montelukast Sodium in Solution and in its Solid State**". A simultaneous measurement of Montelukast and its degradation products was determined

using a selective HPLC method. The HPLC system comprised a reversed phase column (C_{18}) as the stationary phase and a mixture of ammonium acetate buffer of pH 3.5 and methanol (15:85 v/v) as the mobile phase. The UV detection was conducted at 254 nm.

2.2.1.23 Krupa M. Kothekar *et al.,* (2007) reported "**Quantitative Determination of Levofloxacin and Ambroxol Hydrochloride in Pharmaceutical Dosage Form by Reversed - Phase High Performance Liquid Chromatography**". A Hypersil BDS C_{18} column (25 x 4.6mm, 5μm) using a mixture of acetonitrile, methanol and phosphate buffer at pH 5.2, (25:10:65) at a flow rate of 1 ml/ min and were detected at 220 nm.

2.2.1.24 Satinsky Dalibor *et al.,* (2006) reported "**Determination of Ambroxol Hydrochloride, Methyl paraben and Benzoic acid in Pharmaceutical Preparations Based on Sequential Injection Technique Coupled with Monolithic Column**". The HPLC separation Chromolith Speed ROD RP-18e (50 x 4.6 mm, 5 μm) column with acetonitrile tetra hydro furan and water at pH 3.75 (with acetic acid) in 10:10:90 v/v/v at flow rate of 0.48 ml/ min and detection was performed at 245 nm.

2.2.1.25 Dincerzafer *et al.,* (2003) reported "**Quantitative Determination of Ambroxol in Tablets by Derivative UV Spectrophotometric Method and HPLC**". RP-HPLC separation of ambroxol was achieved by C_{18} column with acetonitrile, glacial acetic acid and 0.01 M phosphate buffer at pH 3.12 (40:1:59 v/v/v) and was detected at 252 nm. In first order derivative spectrophotometric method was developed with the analytical wavelength of 255 nm.

2.2.1.26 Grzegorz Bazylak *et al.,* (2003) reported "**Simultaneous High-Throughput Determination of Clenbuterol, Ambroxol and Bromohexine in Pharmaceutical Formulations by HPLC with Potentiometric Detection".** A cyano RP-HPLC with

Uptisphere UP5SCN-25QS silica column (250 x 4.6 mm i.d) eluted with acetonitrile, ethanol and 1.66 mμ water at pH 2.45 with perchloric acid in ratio of 60:2:38 v/v/v and detection was performed potentiometrically.

2.2.1.27 Radhakrishna T *et al*., (2003) reported "**Simultaneous Determination of Montelukast and Loratidine by HPLC and Derivative Spectrophotometric Methods**". The separation was achieved by using symmetry C_{18} column using Acetonitrile and sodium phosphate buffer at pH 3.7 (80:20 v/v), at flow rate of 1 ml/ min. UV detection was performed at 225 nm. In second order derivative spectrophotometry 276.1 nm was selected as wavelength for Loratidine and 359.7 nm for Montelukast Sodium by using methanol as solvent.

2.2.2 Clinical Methods

2.2.2.1 Kang Seung Woo *et al*., (2010) reported "**Enantio Selective Determination of Cetirizine in Human Plasma by Normal – Phase Liquid Chromatography – Atmospheric Pressure Chemical Ionization – Tandem Mass spectrometry**". Enantioselective separation was achieved on a Chiralpak AD-H column in a isocratic mode of elution with n-hexane, ethanol, diethylamine and acetic acid (60:40:0.1:0.1 v/v/v/v). Levocetirizine – D8 was used as internal standard (IS). Levocetirizine and IS were detected by multiple - reaction Monitoring (MRM).

2.2.2.2 Arayne M.S *et al*., (2010) reported "**Simultaneous Determination of Gliquidone, Fexofenadine, Buclizine and Levocetirizine in Dosage Formulation and Human Serum by RP-HPLC.**". A RP-HPLC separation was achieved by methanol and water adjusted to pH 3.5 (80:20 v/v) at a flow rate of 1ml/ min and UV detection was performed at 230 nm.

2.2.2.3 Balasekharareddy Challa *et al.,* (2010) "**Method Development and Validation of Montelukast Sodium in Human Plasma by HPLC Coupled with ESI-MS/MS: Application to a Bioequivalence Study**". Chromatographic separation was performed with YMC-Pack Pro C_{18} (50 x 4.6 mm) S – 3 column with an isocratic mobile phase composed of acetonitrile and 10 mM ammonia formate at pH 4 (80:20 v/v) at a flow rate of 0.8 ml/ min. The detection system was Multiple Reaction Monitoring System (MRM).

2.2.2.4 Xiao – Lin Zhang *et al.,* (2009) reported "**Matrine Determination and Pharmacokinetics in Human Plasma using LC-MS/MS**". A liquid chromatography/ tandem mass spectrometry (LC – MS/MS) method was developed for the determination of matrine in human plasma extracted by isopropanol: ethyl acetate (5:95). Rapid chromatographic separation was achieved in the mobile phase composition of acetonitrile and 5mM aqueous ammonium acetate buffer (70:30 v/v) at a flow rate of 0.20 ml/ min. Detection was carried out using Positive - Ion electroscopy Tandem Mass Spectroscopy on a SCIEX API 3000.

2.2.2.5 Morita M.R. *et al.,* (2008) reported "**Determination of Levocetirizine in Human Plasma by Liquid Chromatography – Electrospray Tandem Mass Spectrometry: Applications to a Bioequivalence Study**". The drug was extracted by Liquid - Liquid Extraction and the separation was achieved using C_{18} column as stationary phase and acetonitrile, water and formic acid (80:19.9:0.1 v/v/v) as mobile phase. The chromatograms are monitored by using positive electrospray source with tandem mass spectrometry.

2.2.2.6 Wanqun Hu *et al.,* (2008) reported "**Rapid and Sensitive Liquid Chromatography Tandem Mass Spectrometry Method for the Quantification of**

Ambroxol in Human Plasma". Chromatographic separation was performed on a Lichrospher CN column with a mobile phase of methanol and water (containing 0.1% formic acid) in 70:30 v/v. The analytes were detected by mass spectrometry with electrospray ionization source in positive selected reaction monitoring mode.

2.2.2.7 Aidong Wen *et al.,* (2008) reported "**Simultaneous Determination of Amoxicillin and Ambroxol in Human Plasma by LC-MS/MS: Validation and Application to Pharmacokinetic Study**". Separation was achieved on a Lichrospher C_{18} column (150 x 4.6 mm, ID, dp 5μm) using methanol and water (containing 0.2% formic acid) as a mobile phase by gradient elution at a flow rate of 1ml/ min. Detection was performed using electrospray ionization in positive ion multiple reaction Monitoring (MRM) mode.

2.2.2.8 Sripalakit Pattana *et al.,* (2008) reported "**A Simple Bioanalytical Assay for Determination of Montelukast Sodium in Human Plasma: Application to a Pharmacokinetic Study**". Chromatographic separation was carried out using a Zorbax eclipse XBD C_8 (150 x 4.6 mm, i.d., 5 μm) with mobile phase consisted of methanol, acetonitrile and 0.04 M disodium hydrogen ortho phosphate buffer at pH 4.9 (22:22:56 v/v/v). The wavelengths of fluorescence detection were set at 350 nm for excitation and 450 nm for emission.

2.2.2.9 Fenli Su *et al*., (2007) reported "**Determination of Ambroxol in Human Plasma by High Performance Liquid Chromatography - Electrospray Ionization Mass Spectrometry (HPLC-MS/ESI)**". Separation was performed by using BDS Hypersil C_{18} (250 x 4.6 mm, 5 μm) column with the mobile phase consisting of 30mM ammonium

acetate buffer (0.4% formic acid) and acetonitrile (64:36 v/v) at a flow rate of 1.2 ml/ min. The detection was carried out with mass spectrophotometer. Fentanyl was used as internal standard (IS).

2.2.2.10 Chauhan.B *et asdl.,* (2006) reported "**A New Liquid - Liquid Extraction Method for Determination of Montelukast in Small Volume Human Plasma using RP-HPLC**". A Kromosil C_8 (150 x 4.6 mm, 5 μm) column was used for separation using isocratic elution technique. The mobile phase used was acetonitrile and 10mM ammonium acetate buffer at pH 3 (65:35) at a flow rate of 1ml/ min and detection was performed by using fluorescence detector at 350 nm as excitation wavelength and 400 nm as emission wave length.

2.2.2.11 Hohyun kim *et al.,* (2003) reported "**Determination of Ambroxol in Human plasma using LC-MS/MS**". A sensitive and selective liquid chromatographic method coupled with tandem mass spectrometry (LC – MS/ MS) was developed for the quantification of Ambroxol in human plasma. Domperidone was used as internal standard. The reconstituted samples were injected into a C_{18} XTerra MS column (2.1 x 30 mm with 3.5 μm particle size) with mobile phase was composed of 20 mM ammonium acetate buffer at pH 8.8 and acetonitrile (90:10 v/v) at a flow rate of 0.25 ml/ min. the mass spectrometer was operated in positive ion mode using turbo electrospray ionization with multiple reaction Monitoring (MRM) mode.

2.2.2.12 Heinanen Maarit *et al.,* (2001) reported "**Validation of an HPLC Method for the Quantification of Ambroxol Hydrochloride and Benzoic acid in Syrup as Pharmaceutical form Stress Test for Stability Evaluation**". Separation of components

were performed by Column symmetry shield RP C_8 (250 x 4.6 mm, 5 μm) as a stationary phase and methanol : 8.5 mM phosphate buffer at pH 2.8 (40:60 v/v) as a mobile phase. The UV detection was performed at 247 nm.

2.2.2.13 Al-Rawithi Sameer *et al.,* (2001) reported "**Expedient Liquid Chromatographic Method with Fluorescence Detection for Montelukast Sodium in Micro - Samples of Plasma**"**.** After simple extraction of plasma a C_8 (4 μm) column was used for separation at 40° C using a single pump. The mobile phase used was acetonitrile and 0.025M sodium acetate buffer at pH 4 (80:20 v/v) and detection was performed at 350 nm as excitation wavelength and 400 nm as emission wavelength.

AIM

AND

PLAN OF WORK

3. AIM AND PLAN OF WORK

3.1 AIM OF WORK

Asthma is the chronic inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction and bronchospasm. The first line of treatment includes single drug therapy and second treatment is the multiple drug therapy. The combined dosage form especially was used to prevent breathing problems during exercise and seasonal allergic rhinitis. There are no adequate methods for the simultaneous estimation of multi drug formulation. So, it is essential to develop newer analytical methods which are simple, precise, accurate, specific, linear and rapid.

In the view of the literature cited, method for estimation of Montelukast sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were reported individually and in combination with other drugs. But no method was reported for the simultaneous estimation of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in bulk and in combined dosage form.

Hence the present work aims to develop simple, precise and accurate methods for the simultaneous estimation of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in bulk and in combined capsule dosage form by UV spectrophotometry and HPTLC. The developed methods were validated as per ICH guidelines.

3.2 PLAN OF WORK

3.2.1 Survey on Literature

A complete literature survey was made on drugs Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride for the various physiochemical properties such as solubility, melting point, storage conditions and analytical techniques individually. These surveys give some basic information of drugs which helps in the process of analytical method development.

3.2.2 Procurement of raw materials and formulation

The bulk drugs Montelukast Sodium was obtained as gift sample from Dr. Reddy's Laboratories Pvt. Ltd, Hyderabad, Levocetirizine Dihydrochloride and Ambroxol hydrochloride were gifted by Madras Pharmaceuticals Pvt. Ltd, Chennai. The formulation RENEA Capsules was purchased from a local Pharmacy in Chennai.

3.2.3 Method development

The solubility of the individual drugs was checked and from the list of solvents the common solvent was selected for both UV spectroscopy and HPTLC must be cheap and readily available.

The various steps involved in the method development are as follows,

UV spectroscopy

1. Selection of appropriate analytical wavelength and selection of suitable method
2. Determination of working concentration range
3. Analysis of synthetic mixture

4. Simultaneous analysis of formulation by using the developed method

HPTLC

1. Determination of suitable detection wavelength
2. Optimization of chromatographic conditions
3. Analysis of formulation

3.2.4 Validation

The developed method should be validated as per ICH and USFDA guidelines. The various parameters of validation are Linearity, Range, Precision, Accuracy, Limit of Detection, Limit of Quantification and Ruggedness.

MATERIALS

AND

METHODS

4. MATERIALS AND METHODS

4.1. MATERIALS USED

4.1.1. Drugs

Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were gifted by Madras Pharmaceuticals Pvt. Ltd., Chennai and Montelukast Sodium was gifted by Dr. Reddy's Laboratories Pvt. Ltd., Hyderabad.

4.1.2 Formulation

RENEA (Shield Healthcare Pvt. Ltd, Chennai) capsule formulation containing Montelukast sodium 10 mg, Levocetirizine Dihydrochloride 5 mg and Ambroxol Hydrochloride 75 mg was purchased from a local Pharmacy in Chennai.

4.1.3. Reagents and Chemicals

All the chemicals used were of analytical grade and HPLC grade procured from Qualigens India Pvt. Ltd, Mumbai and Loba Chemie India Limited, Mumbai. The chemicals used for the study were Methanol (AR grade), Ethyl acetate (AR grade), Toluene (AR grade) and Ammonia Solution 25 % pure (AR grade). Silica gel 60-F_{254} aluminum sheets were procured from, E.Merck and Co, Germany.

4.1.4 Instruments

Different instruments used to carry out the present work,

a) SHIMADZU AUX - 220 Digital balance

b) SHIMADZU - 1700 Double Beam UV - Visible Spectrophotometer with a pair of 10 mm matched quartz cells.

c) ELICO SL - 210 Double Beam UV - Visible Spectrophotometer with a pair of 10 mm matched quartz cells.

d) CAMAG HPTLC System with LINOMAT - 5 Applicator

e) CAMAG TLC Scanner 3 and WINCATS Software

f) ELICO LI - 127 pH meter

g) SOLTEC - Sonica Ultrasonic Cleaner – Model 2200 MH

h) REMI - Centrifuge Apparatus

4.1.5 Specifications of instruments

4.1.5.1 Shimadzu AUX - 220 Digital balance

(*Shimadzu Instruction Manual***)**

Specifications	
Weighing capacity	200 gm
Minimum display	0.1 mg
Standard deviation	≤ 0.1 mg
Operating temperature range	5 to 40°C

4.1.5.2 Double beam UV - visible spectrophotometer

(*Shimadzu and Elico Instruction Manuals*)

Shimadzu UV- 1700 and Elico SL – 210, Cuvettes: 1 cm matched quartz cells

Specification	Shimadzu UV-1700	Elico SL -210
Light source	20 W halogen lamp Deuterium lamp Light source position automatic adjustment mechanism	Tungsten halogen lamp (W) Deuterium lamp (D) Light source position automatic adjustment mechanism
Monochromator	Aberration - correcting concave blazed holographic grating	Concave holographic grating with 1200 lines/ mm
Detector	Silicon photodiode	Photodiode
Stray Light	0.04% or less (220 nm: NAI 10 g/lt) 0.04% or less (340 nm: $NaNo_2$ 50 g/lt)	<0.05% T at 220 nm with NAI 10g/ lt
Measurement Wavelength range	190 ~ 1100 nm	190 ~ 1100 nm
Spectral Band width	1 nm or less (190 to 900 nm)	1.8 nm
Wave length Accuracy	± 0.5 nm on broad automatic wavelength calibration mechanism	± 0.5 nm
Recording range	Absorbance: - 3.99 ~3.99 Abs Transmittance: - 399 ~ 399%	Absorbance: ± 3.000 Abs
Photometric accuracy	± 0.004 Abs (at 1.0 Abs) ± 0.002 Abs (at 0.5 Abs)	0.005 Abs (at 1.0 Abs) 0.010 Abs (at 0.5 Abs)
Operating Temperature Humidity	Temperature range: 15 to 35˚C Humidity range: 35 to 80% (15 to below 35˚C) 35 to 70% (30 to below 35˚C)	Temperature range: 15 to 35˚C Humidity range: 35 to 80% (15 to below 35˚C) 35 to 70% (30 to below 35˚C)

4.1.5.3 HPTLC Instrument Specificatio

4.1.5.3.1 *Pre Coated Silica Gel Plates*

Silica gel 60 - F_{254} aluminum sheets

Plate size - 20 x 10 cm

Material - Silica gel 60-F_{254} aluminum sheets

Pre washing - No

Modification - No

4.1.5.3.2 *Instrument - CAMAG HPTLC System*

Specification	
Spray gas	Inert gas
Solvent used	Methanol
Dosage speed	150 nl/ s
Pre dosage volume	0.2 µl
Syringe size	100 µl
Application volume	10 mm
Band length	8 mm

4.1.5.3.3 *Development – Glass Tank*

Chamber type - Twin trough chamber 20 x 10 cm

Solvent front position - 50.0 mm

Volume - 10.0 ml

Drying device - Oven

Temperature - 60°C

Time - 5 minutes

4.1.5.3.4 *Detection*

CAMAG TLC Scanner 3 TLC scanner 3

Display scaling - Automatic

4.2 METHODS

In the present work, an attempt was made to develop and validate simple, precise and accurate methods for the simultaneous estimation of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in pure and in combined capsule dosage form by UV-Spectrophotometry and HPTLC.

4.2.1 Spectrophotometric Methods

4.2.1.1 Absorbance correction method

If the identity, concentration and absorptivity of the absorbing interferences are known, it is possible to calculate their contribution to the total absorbance of mixture. The concentration of the absorbing component of interest is then calculated from the corrected absorbance (total absorbance minus the absorbance of the interfering substance) in the usual way. The data required for the construction of absorbance corrected for interference are, the λ_{max} of the drug should be found out by using reference standards of the drugs. At one wave length, one of the drugs shows no absorbance. Hence the drug was calculated without any interference. In another wavelength, the absorbance corrected for another drug and the first drug was determined.

4.2.1.2 Selection of solvent

The solubility of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride was determined in variety of solvents as per Indian Pharmacopoeia

standards. From the solubility studies, methanol was chosen as solvent for UV Spectrophotometry.

4.2.1.3 Preparation of standard stock solution

25 mg of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride standard substances were weighed and transferred into 25 ml volumetric flasks separately. Dissolved in methanol and made up to the volume with methanol (1 mg/ ml).

4.2.1.4 Selection of wavelength for absorbance correction method

The standard stock solutions were further diluted with methanol to get the concentration of 10 μg/ml. The solution was scanned between 200 - 400 nm using methanol as blank. The spectra were recorded and overlaid. From the overlain spectra, the λ_{max} of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were found to be 345 nm, 230 nm and 307 nm respectively. At 345 nm, the absorbance of Ambroxol Hydrochloride and Levocetirizine Dihydrochloride are zero. The stability of the drug solution was observed at different time intervals. Montelukast Sodium was stable for 2 hours, Ambroxol hydrochloride was stable for 3 hours and 30 minutes and Levocetirizine Dihydrochloride was stable for 2 hours and 30 minutes. Hence this was selected for the analysis of Montelukast Sodium. At 307 nm Levocetirizine Dihydrochloride absorbance was zero. But both Ambroxol Hydrochloride and Montelukast Sodium had absorbance. To determine the amount of Ambroxol hydrochloride, the absorbance of Montelukast Sodium was corrected at 307 nm. At 230 nm, Montelukast Sodium, Ambroxol Hydrochloride and Levocetirizine Dihydrochloride were exhibiting the absorbance. The absorbance of Montelukast Sodium

and Ambroxol Hydrochloride were corrected and the amount of Levocetirizine Dihydrochloride was determined without any interference.

4.2.1.5 Preparation of calibration graph

4.2.1.5.1 *For Montelukast Sodium*

Working standard solution was prepared by pipetting 1 ml of the standard stock solution into a 50 ml volumetric flask and made up to the volume with methanol to get the concentration 20 µg/ ml. 1 – 6 ml were transferred into a series of 10 ml volumetric flasks and made up to mark with methanol to get the concentration range of 2 – 12 µg/ ml. The absorbance was measured at 345 nm, 307 nm and 230 nm against methanol as blank. The calibration curve was plotted using concentration against absorbance. The procedure was repeated for six times.

4.2.1.5.2 *For Levocetirizine Dihydrochloride*

1 ml of the standard stock solution of Levocetirizine Dihydrochloride was transferred into a 50 ml volumetric flask and made up to the volume with methanol to get the concentration 20 µg/ ml. From that, 0.5 – 6 ml were transferred into a series of 10 ml volumetric flasks and made up to mark with methanol to get the concentration range of 1 – 12 µg/ ml. The absorbance was measured at 230 nm against methanol as blank. The calibration curve was plotted using concentration against absorbance. The procedure was repeated for six times.

4.2.1.5.3 *For Ambroxol Hydrochloride*

Working standard solution was prepared by pipetting 5 ml of the standard stock solution into a 50 ml volumetric flask and made up to the volume with methanol to get the concentration 100 µg/ ml. 1 – 7 ml were transferred into a series of 10 ml volumetric

flasks and made up to mark with methanol to get the concentration range 10 – 70 µg/ ml. The absorbance was measured at 230 nm and 307 nm against methanol as blank. The calibration curve was plotted using concentration against absorbance. The procedure was repeated for six times.

4.2.1.6 Quantification of formulation

Twenty capsules (RENEA containing 10 mg of Montelukast sodium, 5 mg of Levocetirizine Dihydrochloride and 75 mg of Ambroxol Hydrochloride) were weighed accurately. The average weight of each capsule content was found and powdered. The mixed contents of capsule powder equivalent to 25 mg of Ambroxol Hydrochloride was weighed and transferred into 25 ml volumetric flask and added about 20 ml of methanol and sonicated for 15 minutes. The solution was made up to the volume to 25 ml. The solution was centrifuged for 15 minutes at 2000 rpm and filtered through Whatmann filter paper No. 41. From the clear solution, further dilutions were made by diluting 5 ml into 50 ml with methanol. 3 ml was pipetted out into a series of 10 ml volumetric flasks and made up to the mark with methanol to get the concentration of 2 µg/ ml of Levocetirizine Dihydrochloride, 4 µg/ ml of Montelukast Sodium and 30 µg/ ml of Ambroxol Hydrochloride, theoretically. The absorbance was measured at 345 nm, 307 nm and 230 nm for all the solutions. The procedure was repeated for six times. The amount Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were found by applying absorbance correction method.

4.2.1.7 Recovery studies

4.2.1.7.1 *Preparation of raw material stock solution*

125 mg of Montelukast Sodium and Levocetirizine Dihydrochloride were accurately weighed and transferred in to separate 100 ml volumetric flasks, 100 mg of Ambroxol Hydrochloride was accurately weighed and transferred into 10 ml volumetric flask, dissolved in methanol and made up to the mark with methanol to get a concentration of 1.25 mg/ ml of Montelukast Sodium, Levocetirizine Dihydrochloride and 10 mg/ ml of Ambroxol Hydrochloride, respectively.

4.2.1.7.2 *Recovery procedure*

The recovery experiment was done by adding known concentrations of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride working standard to the pre analyzed formulations. The mixed contents of capsule powder equivalent to 25 mg of Ambroxol Hydrochloride was weighed accurately and added 2 ml, 3 ml and 4 ml of Montelukast Sodium, 1 ml, 2 ml and 3 ml of Levocetirizine Dihydrochloride and 2 ml, 2.5 ml and 3 ml of Ambroxol hydrochloride standard solutions were added into a series of 25 ml volumetric flasks, dissolved in methanol and sonicated for 15 minutes. After sonication the solution was made up to 25 ml with methanol. Then the solution were centrifuged for 15 minutes at 2000 rpm and the solutions were filtered through Whatmann filter paper No. 41. From each solution, 5 ml of clear filtrate was transferred into a series of 50 ml volumetric flasks and made up to the volume with methanol. Further dilution was made by pipetting 3 ml into a series of six 10 ml volumetric flasks and made up to the volume with methanol. The absorbances of the resulting solutions were measured at their selected wavelengths (345 nm, 307 nm and 230

nm). The procedure was repeated for three times for each concentration. The amount of each drug recovered from the formulations was calculated for all the drugs by using absorbance correction method.

4.2.1.8 Validation of developed method

4.2.1.8.1 *Linearity*

A calibration curve was plotted between concentration and absorbance. Montelukast Sodium was linear with the concentration range of 2 – 12 μg/ ml at 345 nm, 307 nm and 230 nm Levocetirizine Dihydrochloride was linear with the concentration range of 1 – 12 μg/ ml at 230 nm and Ambroxol Hydrochloride showed the linearity in the range of 10 – 70 μg/ ml at 307 nm and at 230 nm.

4.2.1.8.2 *Accuracy*

Accuracy of the method was confirmed by recovery studies. To the pre analyzed formulations, a known quantity of working standards of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were added and the procedure was followed as per the analysis of formulations. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The percentage RSD and Confidence Interval were calculated.

4.2.1.8.3 *Precision*

The repeatability of the method was confirmed by the analysis of formulations was repeated for six times with the same concentration. The amount of each drug present in the tablet formulations was calculated. The percentage RSD and Confidence Interval were calculated.

The intermediate precision of the method was confirmed by intraday and inter day analysis where the analysis of formulation was repeated three times in the same day and one time on three successive days. The amount of drugs was determined, percentage RSD and Confidence Interval were calculated.

4.2.1.8.4 *Ruggedness*

Ruggedness of the method was confirmed by the analysis of formulation was done with different analysts and with the different instruments. The amount, percentage RSD and Confidence Interval were calculated.

4.2.1.8.5 *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

Preparation of calibration curve from the serial dilutions of standards and was repeated for six times. The Limit of detection and limit of quantification was calculated by using the average value of slope and standard deviation of the intercept.

4.2.2. Derivative Spectrophotometric Method

A simple, accurate, rapid and precise first order derivative spectrophotometric method was developed and validated. The first derivative spectrum is a plot of the rate of change of absorbance with wavelength against wavelength, i.e. a plot of the slope of the fundamental spectrum against wavelength.

4.2.2.1 Selection of solvent

The solubility of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride was determined in variety of solvents as per Indian Pharmacopoeia standards. From the solubility studies, methanol was chosen as solvent for UV Spectrophotometry.

4.2.2.2 Preparation of standard stock solution

25 mg of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride standard substance were weighed and transferred into 25 ml volumetric flasks separately and dissolved in methanol and made up to the volume with methanol (1 mg/ ml).

4.2.2.3 Selection of absorption maxima

The standard stock solutions were further diluted with methanol 10 μg/ ml. The spectra were recorded between 200 - 400 nm using methanol as blank. The spectra were derivatised into first order derivative spectrum. The first order derivative spectra were overlaid and showed that at 365.5 nm, the absorbance of Ambroxol Hydrochloride and Levocetirizine are zero, hence for the analysis of Montelukast Sodium was done at this wavelength. At 248 nm Ambroxol Hydrochloride showed no absorbance but Montelukast Sodium and Levocetirizine Dihydrochloride had absorbance. By correcting the interferent Montelukast Sodium, the absorbance of Levocetirizine Dihydrochloride was determined. At 256.5 nm Levocetirizine Dihydrochloride and Montelukast Sodium had no absorbance but, Ambroxol Hydrochloride had absorbance. Hence it was selected as the analytical wavelength for Ambroxol Hydrochloride. The stability of the drug solution was observed at different time intervals. Montelukast Sodium was stable for 2 hours, Ambroxol hydrochloride was stable for 3 hours and 30 minutes and Levocetirizine Dihydrochloride was stable for 2 hours and 30 minutes.

4.2.2.4 Preparation of calibration graph

4.2.2.4.1 *For Montelukast Sodium*

Working standard solution was prepared by pipetting 1 ml of the standard stock solution into a 50 ml volumetric flask and made up to the volume with methanol to get the concentration 20 µg/ ml. 1 – 6 ml were transferred into a series of 10 ml volumetric flasks and made up to mark with methanol to get the concentration range of 2 – 12 µg/ ml. The absorbance was measured at 365.5 nm and 248 nm against methanol as blank. The calibration curve was plotted using concentration against absorbance. The procedure was repeated for six times.

4.2.2.4.2 *For Levocetirizine Dihydrochloride*

1 ml of the standard stock solution of Levocetirizine Dihydrochloride was transferred into a 50 ml volumetric flask and made up to the volume with methanol to get the concentration 20 µg/ ml. From that 1 – 6 ml were transferred into a series of 10 ml volumetric flasks and made up to mark with methanol to get the concentration range of 1 – 12 µg/ ml. The absorbance was measured at 248 nm against methanol as blank. The calibration curve was plotted using concentration against absorbance. The procedure was repeated for six times.

4.2.2.4.3 *For Ambroxol Hydrochloride*

Working standard solution was prepared by pipetting 5 ml of the standard stock solution into a 50 ml volumetric flask and made up to the volume with methanol to get the concentration 100 µg/ ml. 1 – 7 ml were transferred into a series of 10 ml volumetric flasks and made up to mark with methanol to get the concentration range of 10 – 70 µg/ ml. The absorbance was measured at 256.5 nm against methanol as blank.

The calibration curve was plotted using concentration against absorbance. The procedure was repeated for six times.

4.2.2.5 Quantification of formulation

Twenty capsules (RENEA containing 10 mg of Montelukast sodium, 5 mg of Levocetirizine Dihydrochloride and 75 mg of Ambroxol Hydrochloride) were weighed accurately. The average weight of each capsule content was found and powdered. The mixed contents of capsule powder equivalent to 25 mg of Ambroxol Hydrochloride was weighed and transferred into 25 ml volumetric flask and added about 20 ml of methanol and sonicated for 15 minutes. The solution was made up to the volume to 25 ml. The solution was centrifuged for 15 minutes at 2000 rpm and filtered through Whatmann filter paper No. 41. From the clear solution, further dilutions were made by diluting 5 ml into 50 ml with methanol. 3 ml was pipetted out into a series of 10 ml volumetric flasks and made up to the mark with methanol to get the concentration of 2 μg/ ml of Levocetirizine Dihydrochloride, 4 μg/ ml of Montelukast Sodium and 30 μg/ ml of Ambroxol Hydrochloride, theoretically. The absorbance was measured at 365.5 nm, 256.5 nm and 248 nm for all the solutions. The procedure was repeated for six times. The amount Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were found by applying absorbance correction in derivative method.

4.2.2.6 Recovery studies

4.2.2.6.1 ***Preparation of raw material standard stock solutions***

125 mg of Montelukast Sodium and Levocetirizine Dihydrochloride were accurately weighed and transferred in to separate 100 ml volumetric flasks, 100 mg of Ambroxol Hydrochloride was accurately weighed and transferred into 10 ml volumetric

flask, dissolved in methanol and made up to the mark with methanol to get a concentration of 1.25 mg/ ml of Montelukast Sodium, Levocetirizine Dihydrochloride and 10 mg/ ml of Ambroxol Hydrochloride, respectively.

4.2.2.6.2 ***Recovery procedure***

The recovery experiment was done by adding known concentrations of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride working standard to the pre analyzed formulations. The mixed contents of capsule powder equivalent to 25 mg of Ambroxol Hydrochloride was weighed accurately and added 2 ml, 3 ml and 4 ml of Montelukast Sodium, 1 ml, 2 ml and 3 ml of Levocetirizine Dihydrochloride and 2 ml, 2.5 ml and 3 ml of Ambroxol hydrochloride standard solutions were added into a series of 25 ml volumetric flasks, dissolved in methanol and sonicated for 15 minutes. After sonication the solution was made up to 25 ml with methanol. Then the solution were centrifuged for 15 minutes at 2000 rpm and the solutions were filtered through Whatmann filter paper No. 41. From each solution, 5 ml of clear filtrate was transferred into a series of 50 ml volumetric flasks and made up to the volume with methanol. Further dilution was made by pipetting 3 ml into a series of six 10 ml volumetric flasks and made up to the volume with methanol. Spectra zero order spectra was derivatised to first order spectra and the absorbances of the resulting solutions were measured at their selected wavelengths (365.5 nm, 256.5 nm and 248 nm). The procedure was repeated for three times for each concentration. The amount of each drug recovered from the formulations was calculated for all the drugs by using absorbance correction method in first order derivative spectrophotometry.

4.2.2.7 Validation of developed method

4.2.2.7.1 *Linearity*

A calibration curve was plotted between concentration and absorbance. Montelukast Sodium was linear with the concentration range of 2 – 12 μg/ ml at 365.5 nm, 256.5 nm and 248.0 nm Levocetirizine Dihydrochloride was linear with the concentration range of 2 – 12 μg/ ml at 248.0 nm and Ambroxol Hydrochloride showed the linearity in the range of 10 – 70 μg/ ml at 256.5 nm.

4.2.2.7.2 *Accuracy*

Accuracy of the method was confirmed by recovery studies. To the pre analyzed formulations, a known quantity of working standards of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were added and the procedure was followed as per the analysis of formulations. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The percentage RSD and Confidence Interval were calculated.

4.2.2.7.3 *Precision*

The repeatability of the method was confirmed by the analysis of formulations was repeated for six times with the same concentration. The amount of each drug present in the tablet formulations was calculated. The percentage RSD and Confidence Interval were calculated.

The intermediate precision of the method was confirmed by intraday and inter day analysis where the analysis of formulation was repeated three times in the same day and one time on three successive days. The amount of drugs was determined, percentage RSD and Confidence Interval were calculated.

4.2.2.7.4 *Ruggedness*

Ruggedness of the method was confirmed by the analysis of formulation was done with different analysts and with the different instruments. The amount, percentage RSD and Confidence Interval were calculated.

4.2.2.7.5 *Limit of detection (LOD) and Limit of Quantification (LOQ)*

Preparation of calibration curve from the serial dilutions of standards was repeated for six times. The Limit of detection and limit of quantification was calculated by using the average value of slope and standard deviation of the intercept.

4.2.3. HPTLC Method

4.2.3.1 Choice of Mobile Phase

The initial separation was made with precoated aluminum sheets. The standard and sample solutions of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were spotted and the chromatograms were observed in UV chamber. The following mixture of solvents were tried to optimize the mobile phase.

TRIAL NO	MOBILE PHASE	RATIO
1.	Ethyl acetate: Methanol	8:2
2.	Ethyl acetate: Methanol : 1% Ammonia	8:2:1
3.	Ethyl acetate: Methanol : Toluene: Ammonia	8.5:2.5:1:1
4.	Ethyl acetate: Methanol: Toluene: Ammonia	7:4:1:1
5.	Ethyl acetate: Methanol : Toluene: Ammonia	7:2.5:2.5:1
6.	Ethyl acetate: Methanol : Triethonalamine	5:5:1

From the above list of mobile phases, the mobile phase selected for separation was Ethyl acetate: Methanol: Toluene: Ammonia (7:2.5:2.5:1)

4.2.3.2 Selection of detection wavelength

10 µg/ ml of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were prepared and the spectra were recorded in the range of 200 – 400 nm individually. The spectra were overlaid and from the overlain spectra, 224 nm was selected as detection wavelength, where all the three drugs showed marked absorbance.

4.2.3.3 Optimized Chromatographic Conditions

Stationary phase	:	Silica gel 60-F_{254} aluminum sheets
Mobile phase	:	Ethyl acetate: Methanol: Toluene: 1% Ammonia
Mobile phase ratio	:	7:2.5:2.5:1% v/v
Detection Wavelength	:	UV detection at 224 nm
Development mode	:	Ascending mode
Temperature	:	60° C
Development chamber	:	Twin trough chamber

4.2.3.4 Preparation of Standard Stock Solution

20 mg of Montelukast Sodium, 20 mg of Levocetirizine Dihydrochloride and 75 mg of Ambroxol Hydrochloride were weighed accurately and transferred in to 50 ml volumetric flasks separately. Dissolved in methanol and made up to the volume to 50 ml with methanol. The standard stock solutions contain 400 µg/ ml of Montelukast Sodium, 400 µg/ ml of Levocetirizine Dihydrochloride and 1500 µg/ ml of Ambroxol Hydrochloride. 1 ml of each of the standard stock solution was transferred in to a 10 ml volumetric flask and the solution was diluted to 10 ml with methanol. (This solution contains 40 µg/ ml of Montelukast Sodium, 40 µg/ ml of Levocetirizine Dihydrochloride

and 150 µg/ ml of Ambroxol Hydrochloride) From the solution, 0.5 – 5 µl were applied on silica gel 60- F_{254} aluminum sheets. Concentration range selected were 20 – 200 ng/ µl for Montelukast Sodium, 20 – 200 ng/ µl for Levocetirizine Dihydrochloride and 75 – 750 ng/ µl for Ambroxol Hydrochloride. The calibration graph was plotted using peak area Vs concentration.

4.2.3.5 Quantification of formulation

Twenty capsules (RENEA containing 10 mg of Montelukast Sodium, 5 mg of Levocetirizine Dihydrochloride and 75 mg of Ambroxol Hydrochloride) were accurately weighed and average weight was found. Powdered the mixed contents of the capsule powder, weighed accurately a quantity of the capsule powder equivalent to 75 mg of Ambroxol Hydrochloride and transferred in to a 25 ml volumetric flask, added about 20 ml of methanol, sonicated for 20 minutes and made up to the mark with methanol. The solution was centrifuged at 2000 rpm for 10 minutes and filtered through Whatmann filter paper No.41. 1ml of the above solution was diluted to 10 ml with methanol. 1 µl quantity of the sample was spotted and the chromatogram was recorded. The peak area of the Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were measured and the amount was calculated from the regression equation of the calibration graph. The procedure was repeated for six times.

4.2.3.6 Recovery studies

4.2.3.6.1 ***Preparation of raw material stock solutions***

50 mg of Montelukast sodium, 25 mg of Levocetirizine Dihydrochloride and 375 mg of Ambroxol Hydrochloride raw materials were weighed accurately into 25 ml volumetric flasks separately, dissolved with methanol and made up to the volume with

methanol to get a concentration of 2 mg/ ml, 1 mg/ ml and 15 mg/ ml of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride respectively.

4.2.3.6.2 *Recovery procedure*

The capsule powder equivalent to 75 mg of Ambroxol Hydrochloride was weighed accurately to three separate 50 ml volumetric flasks. To this 4 ml, 5 ml and 6 ml of Montelukast sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride stock solutions were added into 50 ml volumetric flasks. Dissolved in methanol and sonicated for 15 minutes. The solution was then made up to the mark with methanol. The solution was centrifuged for 15 minutes at 2000 rpm and filtered through whatmann filter paper No.41. Further, 1 ml of the stock solution was diluted to a series of six 10 ml volumetric flasks. From this solution, 2 µl quantity of sample were spotted and the chromatogram was recorded. From the peak area, the amounts of drug recovered were calculated for each concentration and repeated for three times.

RESULTS

AND

DISCUSSION

5. RESULTS AND DISCUSSION

The simultaneous estimation of three drugs in a formulation has more advantages such as accurate, less use of reagent and less time requirement for the simultaneous estimation rather than individual estimation of three drugs. Three simple, precise and accurate analytical techniques were developed for the simultaneous estimation of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in combined dosage form. The methods include

1. UV spectroscopic method
   i. Absorption correction method
   ii. First order derivative spectroscopy
2. HPTLC method

5.1 UV SPECTROSCOPIC METHOD

5.1.1 Absorbance Correction Method

The identification of Ambroxol Hydrochloride, Levocetirizine Dihydrochloride and Montelukast Sodium were confirmed by melting point analysis and IR spectral studies (Figures 1 - 3). The solubility of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were determined in variety of solvents as per Indian pharmacopeal standards. Solubility was carried out in polar and non - polar solvents. The common solvents were distilled water, methanol and phosphate buffer at pH 9 for the analysis of these drugs. The solvent (phosphate buffer 9) was not selected as solvent because of its spectral interaction and it is not readily available. Distilled water was not selected as solvent due to formation of opalescence with Montelukast Sodium during solubility. Hence it was avoided to be used as solvent. Finally, methanol was

selected as solvent on account of its solubility factor and cut off wavelength. The solubility profile of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride are given in table 1, 2 and 3 respectively.

The sample solution of 10µg/ ml of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in methanol were prepared individually and the solutions were scanned between 200 – 400 nm by using methanol as blank. From the overlain spectra by observing the spectral characteristics absorbance correction method was selected for the simultaneous estimation of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in combined dosage form. In this method λ_{max} of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were selected as analytical wavelengths i.e. 345 nm, the λ_{max} of Montelukast Sodium 307 nm, the λ_{max} of Ambroxol Hydrochloride and 230 nm the λ_{max} of Levocetirizine Dihydrochloride. At 345 nm Montelukast Sodium has absorbance but Ambroxol Hydrochloride and Levocetirizine Dihydrochloride has zero absorption. Hence the amount of Montelukast Sodium was calculated by using its absorptivity value at 345 nm without any interference. At 307 nm Levocetirizine Dihydrochloride absorbance was zero. But both Ambroxol Hydrochloride and Montelukast Sodium had absorbance. To determine the amount of Ambroxol Hydrochloride the absorbance of Montelukast Sodium was corrected at 307 nm. At 230 nm, Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were exhibiting the absorbance values. The absorbance of Montelukast Sodium and Ambroxol Hydrochloride were corrected for interference and the amount of Levocetirizine Dihydrochloride was determined without any interference. The overlain spectrum of Montelukast Sodium, Levocetirizine

Dihydrochloride and Ambroxol Hydrochloride was shown in figure 4. The stability of the drug solution was observed at different time intervals. Montelukast Sodium was stable for 2 hours, Ambroxol Hydrochloride was stable for 3 hours and 30 minutes and Levocetirizine Dihydrochloride was stable for 2 hours and 30 minutes.

Aliquots of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were prepared were prepared in concentration range of 2 – 12 µg/ ml for Montelukast Sodium, 1 – 12 µg/ ml for Levocetirizine Dihydrochloride and 10 – 70 µg/ ml for Ambroxol Hydrochloride. The calibration curve was plotted with absorbance versus concentration for the three drugs. The optical characteristics such as correlation coefficient slope, intercept, LOD and LOQ were calculated and regression equation was constructed for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride. The correlation coefficient was found to be 0.9994 for Montelukast Sodium at 345 nm, 0.9990 and 0.9994 for Montelukast Sodium and Ambroxol Hydrochloride at 307 nm and 0.9991, 0.9994 and 0.9993 for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride at 230 nm respectively. At 345 nm the LOD and LOQ were found to be 0.6755 µg/ ml and 2.04715 µg/ ml for Montelukast Sodium. At 307 nm the LOD and LOQ were found to be 0.4205 µg/ ml and 1.26149 µg/ ml for Montelukast sodium and 1.61590 µg/ ml and 4.89667 µg/ ml for Ambroxol Hydrochloride. At 230 nm, the LOD and LOQ were found to be 0.4130 µg/ ml and 1.2515 µg/ ml for Montelukast Sodium, 0.05280 µg/ ml and 0.1600829 µg/ ml for Levocetirizine Dihydrochloride and 1.1978 µg/ ml and 3.6297 µg/ ml for Ambroxol Hydrochloride. The correlation coefficient values at all the selected wavelengths are found to be above 0.999. Hence the selected concentrations are

linear and obeyed Beer's law. The calibration graphs for Montelukast Sodium at 345 nm, 307 nm and 230 nm are shown in figure 5, 6 and 7 respectively. The calibration graphs for Ambroxol Hydrochloride at 307 nm and 230 nm are shown in figure 8 and 9. The calibration graph for Levocetirizine Dihydrochloride at 230 nm is in figure 10 shown optical characteristic at 345 nm, 307 nm and 230 nm are shown in tables 4, 5 and 6 respectively.

The capsule formulation, RENEA (containing 5 mg of Levocetirizine Dihydrochloride, 10 mg of Montelukast Sodium and 75 mg of Ambroxol Hydrochloride) was selected for analysis. The drugs Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride are in ratio 2:1:15 in the formulation. The percentage purity of drugs in the formulation was found to be 100.45 ± 0.6253 for Montelukast Sodium, 100.38 ± 1.7426 for Levocetirizine Dihydrochloride and 100.32 ± 1.1773 for Ambroxol Hydrochloride. The precision of the method was confirmed by the repeated analysis of the formulation for six times. The percentage RSD was calculated. The percentage RSD of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were found to be 0.6224, 1.7359 and 1.1735 respectively. Confidence Interval (95 %) were found to be 99.79 – 101.10 for Montelukast Sodium, 98.55 – 102.20 for Levocetirizine Dihydrochloride and 99.08 – 101.55 for Ambroxol hydrochloride, respectively. The low RSD values suggest that the method has good precision. The results are shown in table 7.

Further, precision of the method was confirmed by Intraday and Inter day analysis. Intraday and Interday analysis of formulation was done on three times on same day and one time on three consecutive days. The percentage RSD for the Intraday and

Interday precision was found to be 0.2310 and 0.2651 for Montelukast Sodium, 0.6116 and 0.3376 for Levocetirizine Dihydrochloride and 0.8731 and 0.0300 for Ambroxol Hydrochloride. The low RSD values suggest that the precision of the method was further confirmed. Confidence Interval (95 %) were found to be 99.71 – 100.20 and 99.52 – 100.07 for Montelukast Sodium, 101.4 – 102.75 and 102.23 – 103.61 for Levocetirizine Dihydrochloride and 100.86 – 102 73 and 101.51 – 101.58 for Ambroxol hydrochloride, for intraday and interday, respectively. The results of analysis are given in table 8.

The ruggedness of the method was validated by using different analysts and different instruments. The percentage RSD for analyst 1 and analyst 2 were found to be 0.6224 and 0.4666 for Montelukast Sodium, 1.7359 and 1.8957 for Levocetirizine Dihydrochloride and 1.1735 and 0.4660 for Ambroxol Hydrochloride respectively. The percentage RSD value for the instrument 1 and instrument 2 was found to be 0.6026 and 1.0188 for Montelukast Sodium, 0.8306 and 0.7499 for Levocetirizine Dihydrochloride and 1.2706 and 1.1656 for Ambroxol Hydrochloride respectively. Confidence Interval (95 %) were found to be 98.69 – 99.94 and 98.91 – 101.05 for Montelukast Sodium, 100.61 – 102.38 and 101.08 – 102.68 for Levocetirizine Dihydrochloride and 98.07 – 101.34 and 98.43 – 100.87 for Ambroxol hydrochloride, for Instrument 1 and Instrument 2 respectively. Confidence Interval (95 %) were found to be 99.79 – 101.1 and 99.11 – 100.08 for Montelukast Sodium, 98.55 – 102.2 and 99.71 – 103.86 for Levocetirizine Dihydrochloride and 99.08 – 101.55 and 101.18 – 102.17for Ambroxol hydrochloride, for analyst 1 and analyst 2, respectively. The results of the analysis are given in table 9 and 10 respectively.

The accuracy of the method was confirmed by recovery studies. To the pre analysed formulation a known quantity of the raw material is added and the percentage recovery was calculated. The percentage of raw material added was 80 %, 100 % and 120 % for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride. The percentage recovery was found to be in the range of 98.45 to 100.35% for Montelukast Sodium, 97.77 to 102.42 % for Levocetirizine Dihydrochloride and 98.27 to 102.57 % for Ambroxol Hydrochloride. Percentage RSD values were found to be 0.5286, 1.4463 and 0.5541 for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride, respectively. The low percentage RSD indicated that there was no interference due to excepients used in formulation. Hence, the accuracy of the method was conformed. The data for recovery studies are given in table 11, 12 and 13.

5.1.2 First Order Derivative Spectrophotometry

A simple, precise, accurate and rapid derivative spectrophotometric method was developed and validated. From the overlain first order derivative spectrum (figure 11), 365.5 nm was selected for estimation of Montelukast Sodium without any interference because of both Ambroxol Hydrochloride and Levocetirizine Dihydrochloride have zero absorbance at 365.5 nm. At 256.5 nm Ambroxol Hydrochloride has absorbance where, both Montelukast Sodium and Levocetirizine Dihydrochloride have zero absorbance. Hence Ambroxol Hydrochloride can be estimated without any interference. At 248 nm Ambroxol Hydrochloride has zero absorbance, where Levocetirizine Dihydrochloride and Montelukast Sodium had absorbance. By correcting the interference due to

Montelukast Sodium at 248 nm, the Levocetirizine Dihydrochloride could be estimated. Hence all drugs can be estimated without prior separation.

Different aliquots of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were prepared in the concentration range were prepared in concentration range of 2 – 12 µg/ ml, 2 – 12 µg/ ml and 10 – 70 µg/ ml respectively. The zero order spectra was derivatised to first order derivative spectra and the absorbance were measured at 365.5 nm and 248 nm for Montelukast Sodium, 256.5 nm for Ambroxol Hydrochloride and 248 nm for Levocetirizine Dihydrochloride. The calibration curve was constructed with absorbance versus concentration for three drugs. The optical characteristics such as correlation coefficient slope, intercept, LOD and LOQ were calculated and regression equation was constructed for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride. The correlation coefficient was found to be 0.9992 for Montelukast Sodium at 365.5 nm, 0.9993 and 0.9993 for Montelukast Sodium and Levocetirizine Dihydrochloride at 248 nm and 0.9989 for Ambroxol Hydrochloride at 256.5 nm respectively. At 365.5 nm the LOD and LOQ were found to be 0.6961 µg/ ml and 2.0883 µg/ ml for Montelukast Sodium, at 248 nm the LOD and LOQ were found to be 0.4483 µg/ ml and 1.3587 µg/ ml for Levocetirizine Dihydrochloride and 0.4568 µg/ ml and 1.3844 µg/ ml for Montelukast Sodium. At 256.5 nm the LOD and LOQ were found to be 1.7282 µg/ ml and 5.2371 µg/ ml for Ambroxol Hydrochloride. The correlation coefficient values at all the selected wavelengths are found to be around 0.999. Hence the selected concentrations are linear and obeyed beer's law. The calibration graphs for Montelukast Sodium at 365.5 nm and 248 nm are shown in figure 12 and 13 respectively. The calibration graph for Ambroxol Hydrochloride at

256.5 nm is shown in figure 14. The calibration graph for Levocetirizine Dihydrochloride at 248 nm is shown in figure 15. The optical characteristics at 365.5 nm, 248 nm and 256.5 nm are shown in tables 14, 15 and 16 respectively.

The capsule formulation, RENEA (containing 5 mg of Levocetirizine Dihydrochloride, 10 mg of Montelukast Sodium and 75 mg of Ambroxol Hydrochloride) was selected for analysis. The drug Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride are in ratio 2:1:15 in the formulation. The percentage purity of drugs in the formulation was found to be 99.2 ± 0.8854 for Montelukast Sodium, 101.21 ± 0.8900 for Levocetirizine Dihydrochloride and 99.82 ± 1.0461 for Ambroxol Hydrochloride. The precision of the method was confirmed by the repeated analysis of the formulation for six times. The percentage RSD was calculated and the percentage RSD of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were found to be 0.8925, 0.8794 and 1.0479 respectively. Confidence Interval (95 %) were found to be 98.27 – 100.12 for Montelukast Sodium, 100.27 – 102.14 for Levocetirizine Dihydrochloride and 98.72 – 100.91 for Ambroxol hydrochloride, respectively. The low percentage RSD values suggest that the method has good precision. The results were shown in table 17.

The intermediate precision is checked by Intraday and Interday analysis. Intraday and Interday analysis of formulation was done on three times on same day and one time on three consecutive days. The percentage RSD for the Intraday and Inter day precision was found to be 0.5296 and 0.0116 for Montelukast Sodium, 0.7579 and 0.1123 for Levocetirizine Dihydrochloride and 1.2816 and 1.2361 for Ambroxol Hydrochloride. Confidence Interval (95 %) were found to be 98.63 – 99.74 and 99.67 – 99.90 for

Montelukast Sodium, 100.50 – 101.11 and 102.60 – 102.81 for Levocetirizine Dihydrochloride and 99.69 – 101.40 and 99.15 – 101.76 for Ambroxol hydrochloride, for intraday and interday, respectively. The low percentage RSD suggests that the methods have a good precision. The results of the analysis are given in table 18.

The ruggedness of the method was validated by using different analysis and different instruments. The percentage RSD for analyst 1 and analyst 2 were found to be 0.8925 and 0.7136 for Montelukast Sodium, 0.8794 and 1.8801 for Levocetirizine Dihydrochloride and 1.0479 and 0.7363 for Ambroxol Hydrochloride respectively. The percentage RSD value for the instrument 1 and instrument 2 were found to be 0.7053 and 1.1018 for Montelukast Sodium, 1.7752 and 0.8363 for Levocetirizine Dihydrochloride and 0.8063 and 1.1839 for Ambroxol Hydrochloride, respectively. Confidence Interval (95 %) were found to be 98.61 – 100.08 and 98.99 – 101.3 for Montelukast Sodium, 99.02 – 102.77 and 100.30 – 102.07 for Levocetirizine Dihydrochloride and 99.55 – 101.24 and 99.59 – 102.1 for Ambroxol hydrochloride, for Instrument 1 and Instrument 2 respectively. Confidence Interval (95 %) were found to be 98.27 – 100.12 and 98.41 – 99.90 for Montelukast Sodium, 100.27 – 102.14 and 99.16 – 103.11 for Levocetirizine Dihydrochloride and 98.72 – 100.19 and 98.68 – 100.21 for Ambroxol hydrochloride, for analyst 1 and analyst 2, respectively. The results of analysis are given in table 19 and 20 respectively.

The accuracy of the method was confirmed by recovery studies. To the pre analysed formulation a known quantity of the raw material is added and the percentage recovery was calculated. The percentage of raw material added was 80%, 100% and 120% for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol

Hydrochloride. The percentage recovery was found to be in the range of 97.68 to 100 % for Montelukast Sodium, 98.46 to 103.4 % for Levocetirizine Dihydrochloride and 96.89 to 99.55 % for Ambroxol Hydrochloride. RSD values were found to be 0.6684, 1.1912 and 0.8238 for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride, respectively. The low percentage RSD indicated that there was no interference due to excepients used in formulation. Hence, the accuracy of the method was conformed. The data for recovery studies are given in table 21, 22 and 23.

5.2 HPTLC METHOD

An effort was made to develop a simple, precise and accurate method for the simultaneous estimation of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in bulk and in combined dosage form by HPTLC method.

The initial separation was mode using various mobile phase using ethylacetate, methanol, ammonia, triethonalamine and toluene in different combinations. Finally, ethylacetate: methanol: toluene: ammonia in the ratio of 7:2.5:2.5:1 v/v was selected for the method. Since, all the three drugs were eluted and separated with good resolution between them. The optimized chromatograms are shown in figure 16, 17 and 18 for Montelukast Sodium, Ambroxol Hydrochloride and Levocetirizine Dihydrochloride, respectively.

The detection wavelength was assigned from the spectral characters of all the three the drugs by comparing with standard solution and sample solution. 224 nm was selected as detection wavelength for the analysis. This is shown in figure 19. 400 µg/ ml of Montelukast Sodium, 400 µg/ ml of Levocetirizine Dihydrochloride and 1500 µg/ ml

of Ambroxol Hydrochloride were prepared in methanol. Form the stock solution concentration range of 20 to 200 µg/ ml for Montelukast Sodium, 20 to 200 µg/ ml for Levocetirizine Dihydrochloride and 75 to 750 µg/ ml for Ambroxol Hydrochloride were prepared and developed in twin through chamber. The linearity chromatograms are shown in figure 20 to 29. The calibration graph was plotted with concentration versus peak area and the correlation coefficient was found to be 0.9992, 0.9990 and 0.9993 for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride, respectively. The calibration graph for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride are shown in figure 30, 31 and 32 respectively. The optical characteristic such as LOD, LOQ, slope, intercept, regression equation and correlation coefficient are shown in table 24.

The capsule content equivalent to 75 mg of Ambroxol Hydrochloride was taken and dissolved in 20 ml of methanol, sonicated for 20 minutes and made up to 25 ml with methanol. The solution was centrifuged at 2000 rpm for 10 min and the filtered through whatmann filter paper No.41. This solution was diluted to get a final solution containing 40 µg/ ml of Montelukast Sodium, 20 µg/ ml of Levocetirizine Dihydrochloride and 300 µg/ ml of Ambroxol Hydrochloride theoretically. The chromatogram was developed and peak areas were noted. The percentage purity of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were found to be 99.97 ± 0.4881, 101.89 ± 1.6881 and 100.02 ± 0.5487 respectively. The percentage RSD values for Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride are 0.4882, 1.6567 and 0.5486, respectively. Confidence Interval (95 %) were found to be 99.45 – 100.48 for Montelukast Sodium, 100.11 – 103.66 for Levocetirizine

Dihydrochloride and 98.39 – 101.64 for Ambroxol hydrochloride, respectively. The chromatograms are given in figure 33 to 38.The data is given in table 25.

The intermediate precision is checked by Intraday and Inter day analysis. The percentage RSD for Intraday and Interday precision was found to be 0.2379 and 0.5234 for Montelukast Sodium, 1.1657 and 0.3168 for Levocetirizine Dihydrochloride and 0.2508 and 0.3269 for Ambroxol Hydrochloride. Confidence Interval (95 %) were found to be 99.71 – 100.7 and 99.61 – 100.62 for Montelukast Sodium, 99.44 - 101.95 and 101.91 – 102.31 for Levocetirizine Dihydrochloride and 100.06 – 101.03 and 99.13 – 100.12 for Ambroxol hydrochloride, for intraday and interday, respectively. The low percentage RSD suggests that the method have a good precision. The results of the analysis are given in table 26.

The accuracy of the method is confirmed by recovery analysis. To the pre analysed formulation known quantities of standard drugs were added at three different concentrations. The amount of Levocetirizine Dihydrochloride was in the range of 96.34 to 100.73 %, 96.90 to 100 % for Montelukast Sodium and 98.17 to 100.35 % for Ambroxol Hydrochloride. RSD values were found to be 1.1517, 1.0042 and 0.5977 for Levocetirizine Dihydrochloride, Montelukast Sodium and Ambroxol Hydrochloride, respectively. The low RSD values indicate that there are no interference of excepients during the analysis. The peaks of the developed chromatogram are given in figure 39 to 41. The data of recovery analysis are given in table 27, 28 and 29.

SUMMARY

AND

CONCLUSION

6. SUMMARY AND CONCLUSION

Three simple, rapid and accurate methods were developed for the simultaneous estimation of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in bulk and in combined dosage forms.

The methods developed were

1. UV spectrophotometric method
    i. Absorption correction method
    ii. First order derivative spectroscopy
2. HPTLC method

6.1 UV SPECTROPHOTOMETRIC METHOD

6.1.1 Absorbance Correction Method

From the solubility profile of the three drugs methanol was chosen as the common solvent for the simultaneous estimation of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride.

A 10 µg/ ml solution of all the three drugs and spectra were recorded from the overlain spectra, 345 nm, 307 nm and 230 nm were selected for the analysis. The concentration of 2 to 12 µg/ ml for Montelukast Sodium, 1 to 12 µg/ ml for Levocetirizine Dihydrochloride and 10 to 70 µg/ ml for Ambroxol Hydrochloride were prepared and the absorbance were measured at 345 nm, 307 nm and 230 nm. The calibration graph was plotted. The correlation coefficients for all the three drugs are more than 0.999. The optical parameters like the slope, intercept, correlation coefficient, LOD, LOQ, sandell's sensitivity and molar absorptivity were calculated.

RENEA capsule containing 10 mg of Montelukast Sodium, 5 mg of Levocetirizine Dihydrochloride and 75 mg of Ambroxol Hydrochloride was taken for the analysis. The percentage of drugs in the formulation was found to be 100.45 ± 0.6253 for Montelukast Sodium, 100.38 ± 1.7426 for Levocetirizine Dihydrochloride and 100.32 ± 1.1773 for Ambroxol Hydrochloride. Further the method was validated for precision, accuracy and ruggedness. The precision was confirmed by low percentage RSD values for Intraday and Interday analysis. The percentage RSD for Intraday and Inter day analysis were found to be 0.2310 and 0.2651 for Montelukast Sodium, 0.6116 and 0.3376 for Levocetirizine Dihydrochloride and 0.8731 and 0.0300 for Ambroxol Hydrochloride, respectively. The ruggedness was confirmed by different instrument analysis and by performing the analysis with different analysts. The percentage RSD for the analyst1 and analyst 2 found to be 0.6224 and 0.4666 for Montelukast Sodium, 1.7359 and 1.8957 for Levocetirizine Dihydrochloride and 1.1735 and 0.4660 for Ambroxol Hydrochloride respectively. The percentage RSD for instrument 1 and instrument 2 are 0.6026 and 1.0188 for Montelukast Sodium, 0.8306 and 0.7499 for Levocetirizine Dihydrochloride and 1.2706 and 1.1656 for Ambroxol Hydrochloride respectively. The percentage recovery was found to be in the range of 98.45 to 100.35 % for Montelukast Sodium, 98.77 to 102.47 % for Levocetirizine Dihydrochloride and 98.27 to 102.57 % for Ambroxol Hydrochloride.

6.1.2 First Order Derivative Spectrophotometry

A 10 µg/ ml solution of all the three drugs and spectra were recorded. The zero order spectra were derivatised to first order spectra and form the overlain spectra, 365.5 nm, 256.5 nm and 248 nm were selected for the analysis.

The concentration of 2 to 12 µg/ ml for Montelukast Sodium, 2 to 12 µg/ ml for Levocetirizine Dihydrochloride and 10 to 70 µg/ ml for Ambroxol Hydrochloride were prepared and the absorbance were measured at 365.5 nm, 256.5 nm and 248 nm. The calibration graph was plotted. The optical parameters like the slope, intercept, correlation coefficient, LOD, LOQ, sandell's sensitivity and molar absorptivity were calculated. RENEA capsules containing 10 mg of Montelukast Sodium, 5 mg of Levocetirizine Dihydrochloride and 75 mg of Ambroxol Hydrochloride was taken for the analysis. The percentage of drugs in the formulation was found to be 99.2 ± 0.8854 of Montelukast Sodium, 101.21 ± 0.8900 for Levocetirizine Dihydrochloride and 99.82 ± 1.0461 for Ambroxol Hydrochloride.

Further the method was validated for precision, accuracy and ruggedness. The precision was confirmed by low percentage values for Intraday and Inter day analyst. The percentage RSD for Intraday and Interday analysis were found to be 0.5296 and 0.0116 for Montelukast Sodium, 0.7579 and 0.1123 for Levocetirizine Dihydrochloride and 1.2816 and 1.2361 for Ambroxol Hydrochloride respectively. The ruggedness was confirmed by different instrument analysis and by performing the analysis with different analysts. The percentage RSD for the analyst 1 and analyst 2 were found to be 0.8925 and 0.7136 for Montelukast Sodium, 0.8794 and 1.8801 for Levocetirizine Dihydrochloride and 1.0479 and 0.7363 for Ambroxol Hydrochloride, respectively. The percentage recovery was found to be in the range of 97.68 – 100 % for Montelukast Sodium, 98.46 - 103.4 % for Levocetirizine Dihydrochloride and 96.89 - 99.55% for Ambroxol Hydrochloride.

6.2 HPTLC

A simple and rapid HPTLC method was developed for the simultaneous estimation Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in bulk and in combined dosage form.

The mobile phase consisting of ethyl acetate: methanol: toluene: ammonia in the ratio of 7: 2.5: 2.5: 1 % v/v was selected for analysis. With the optimized conditions, the linearity range was fixed as 20 - 200 µg/ ml for Montelukast Sodium, 20 - 200 µg/ ml for Levocetirizine Dihydrochloride and 75 - 750 µg/ ml for Ambroxol Hydrochloride. The correlation coefficient for all three drugs was found to be more than 0.999. The optical characters such as the LOD, LOQ, slope, and intercept were calculated.

The capsule dosage form RENEA was selected for the analysis. The percentage purity was found to be 99.97 ± 0.4881 for Montelukast Sodium, 101.8933 ± 1.6881 for Levocetirizine Dihydrochloride and 100.02 ± 0.5487 for Ambroxol Hydrochloride. The percentage RSD was found to be 0.4882 for Montelukast Sodium, 1.6567 for Levocetirizine Dihydrochloride and 0.5486 for Ambroxol Hydrochloride.

The precision of the method was confirmed by low percentage RSD values for Intraday and Interday analysis. The percentage RSD for Intraday and Inter day analysis were found to be 0.2379 and 0.5234 for Montelukast Sodium, 1.1657 and 0.3168 for Levocetirizine Dihydrochloride and 0.2508 and 0.3269 for Ambroxol Hydrochloride respectively.

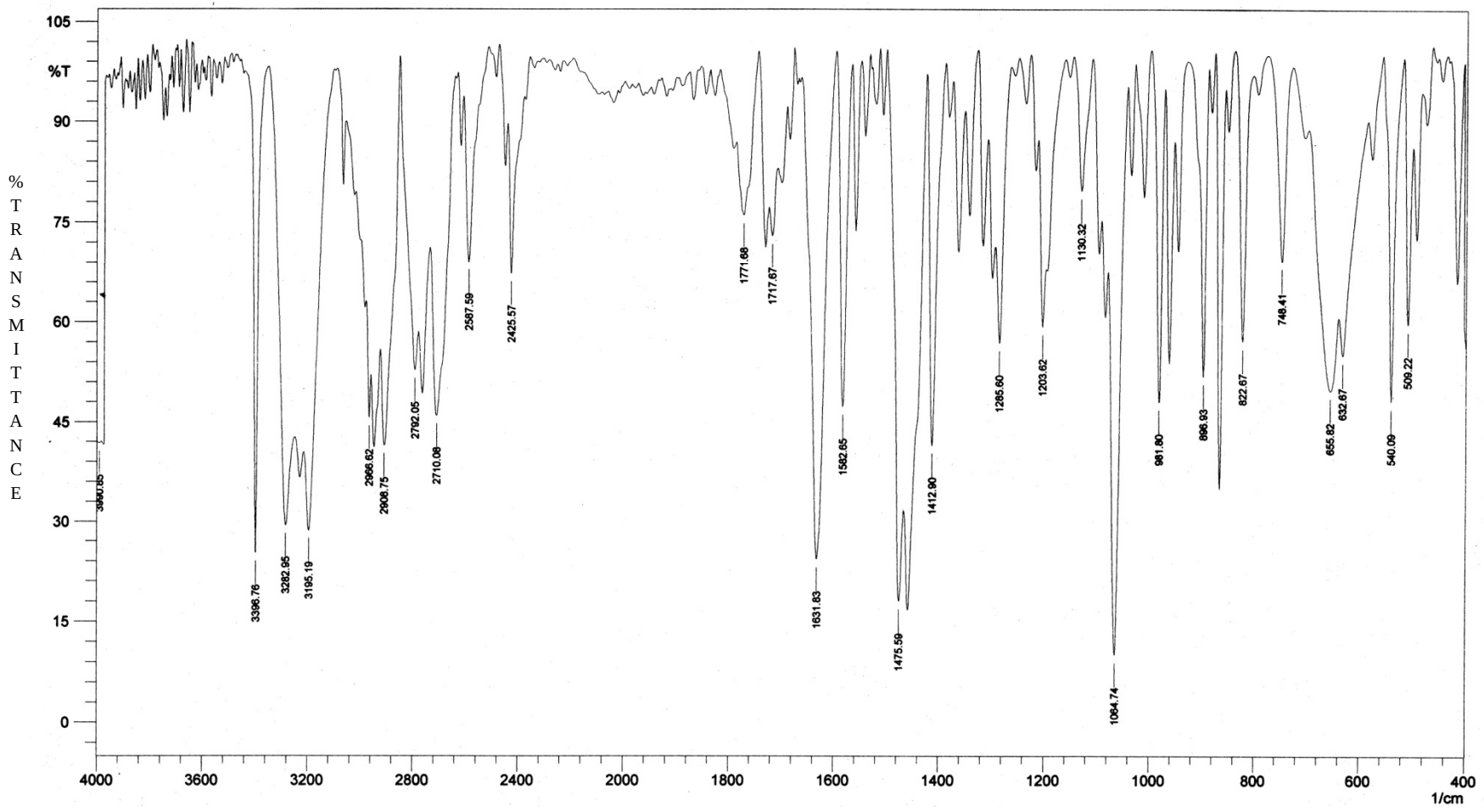
The recovery study confirms the accuracy of the method. The percentage recovery of Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride were in the range of 96.90 to 100 % for Montelukast Sodium,

96.34 to 100.73 % for Levocetirizine Dihydrochloride and 98.17 to 100.35 % for Ambroxol Hydrochloride.

The three methods were found to be accurate, precise and rapid for the simultaneous estimation of the drugs. This was confirmed by percentage RSD values. The spectrophotometric method is found to be economical when compared to HPTLC method. But the HPTLC method is found to be more sensitive compared to spectroscopic methods. The low percentage RSD value in the recovery studies suggest that the excepients do not interfere in the analysis of the formulation and all methods are accurate. HPTLC method is found to be more sensitive than the other methods. Because of its linearity range, LOD and LOQ were less in HPTLC method. Hence it is suggested that the three methods can be applied successfully for the routine quality control analysis of the drugs Montelukast Sodium, Levocetirizine Dihydrochloride and Ambroxol Hydrochloride in bulk and in combine formulation and the obtained result will be presented elsewhere.

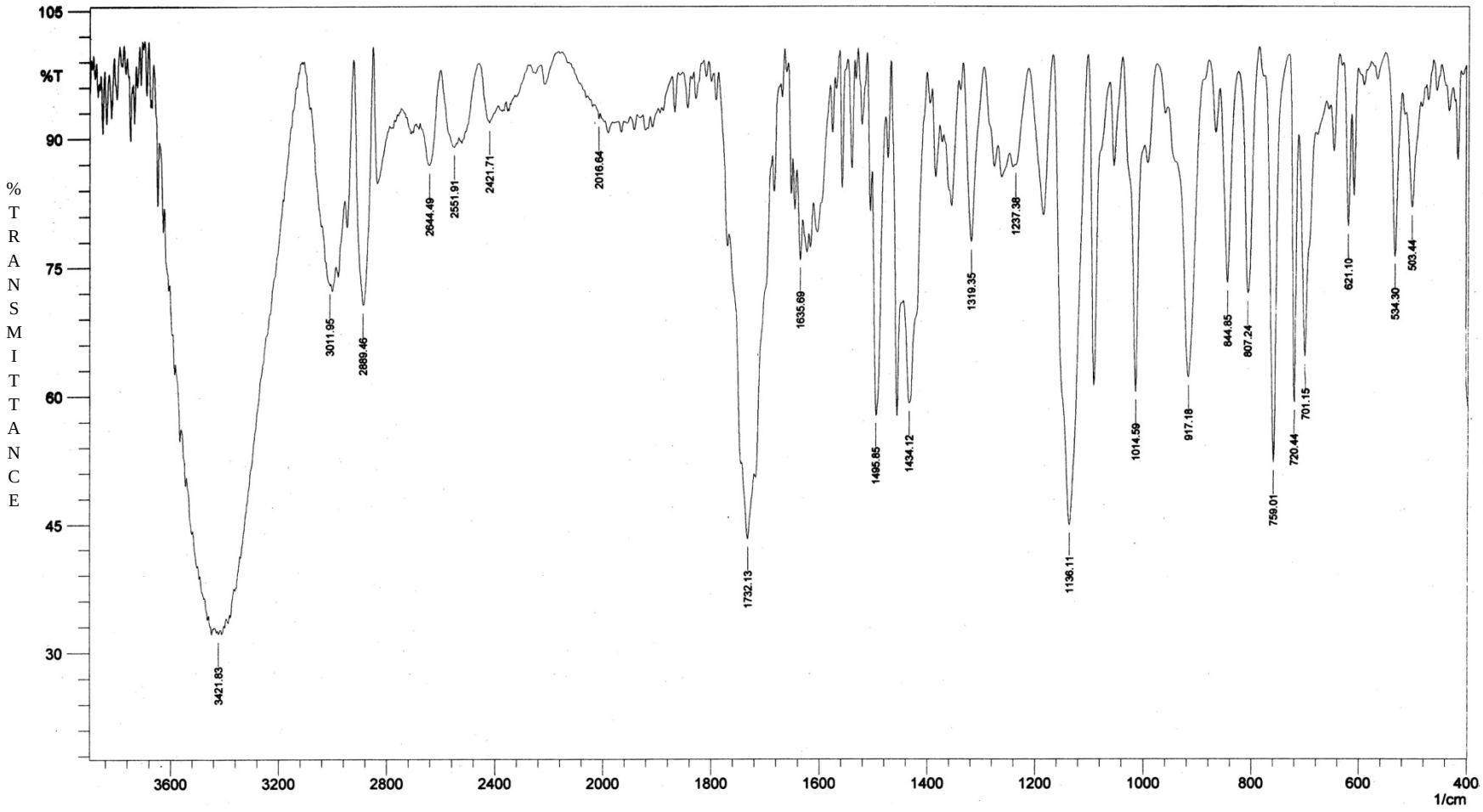
FIGURES

FIGURE – 1 IR SPECTRUM OF AMBROXOL HYDROCHLORIDE



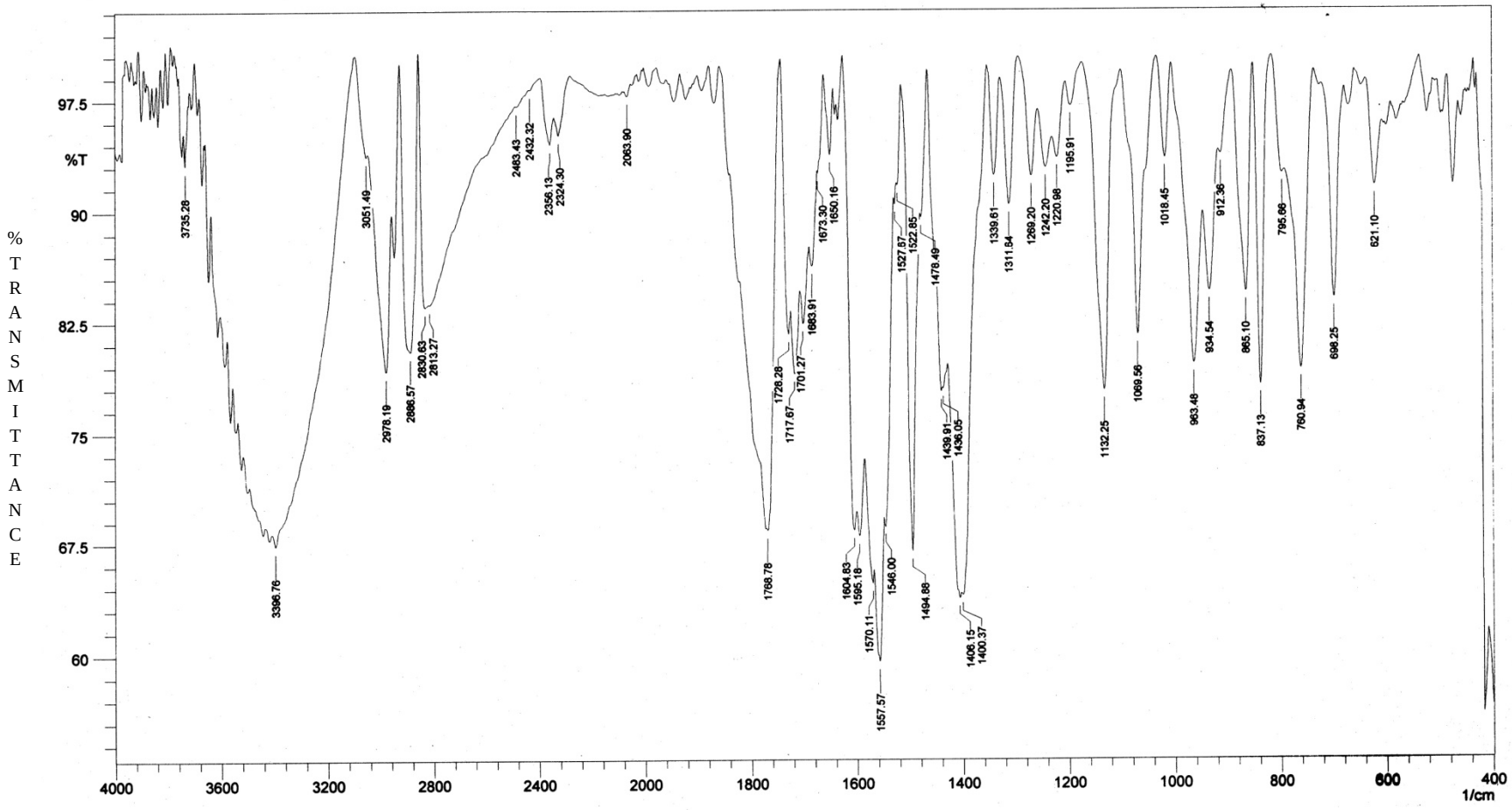
Wave number (cm^{-1})

FIGURE – 2 IR SPECTRUM OF LEVOCETIRIZINE DIHYDROCHLORIDE



Wave number (cm^{-1})

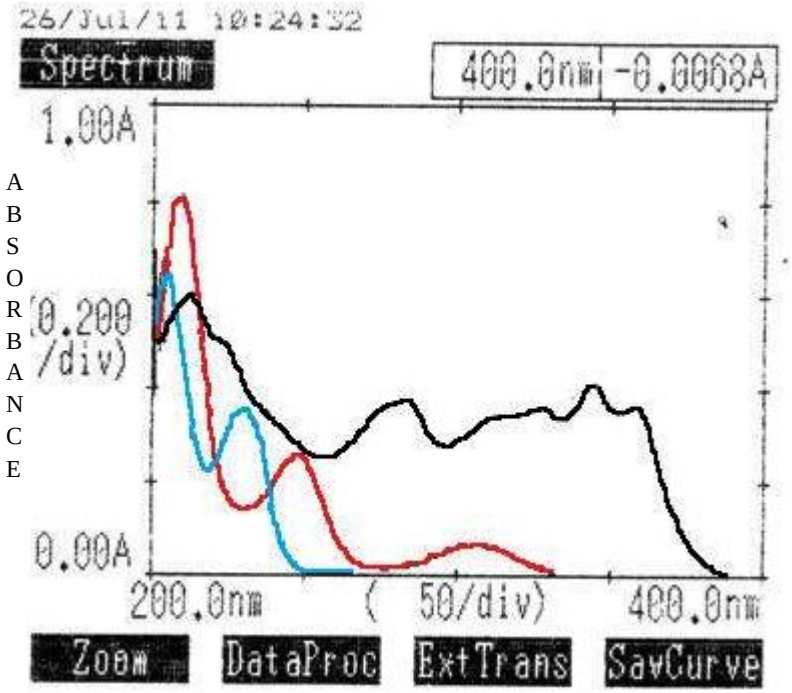
FIGURE – 3 IR SPECTRUM OF MONTELUKAST SODIUM



Wave number (cm^{-1})

FIGURE - 4

UV OVERLAIN SPECTRUM OF MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE IN METHANOL



WAVELENGTH (nm)

- **Levocetirizine Dihydrochloride**
- **Ambroxol hydrochloride**
- **Montelukast Sodium**

FIGURE - 5

CALIBRATION CURVE OF MONTELUKAST SODIUM IN

METHANOL AT 345 nm

(ABSORBANCE CORRECTION METHOD)

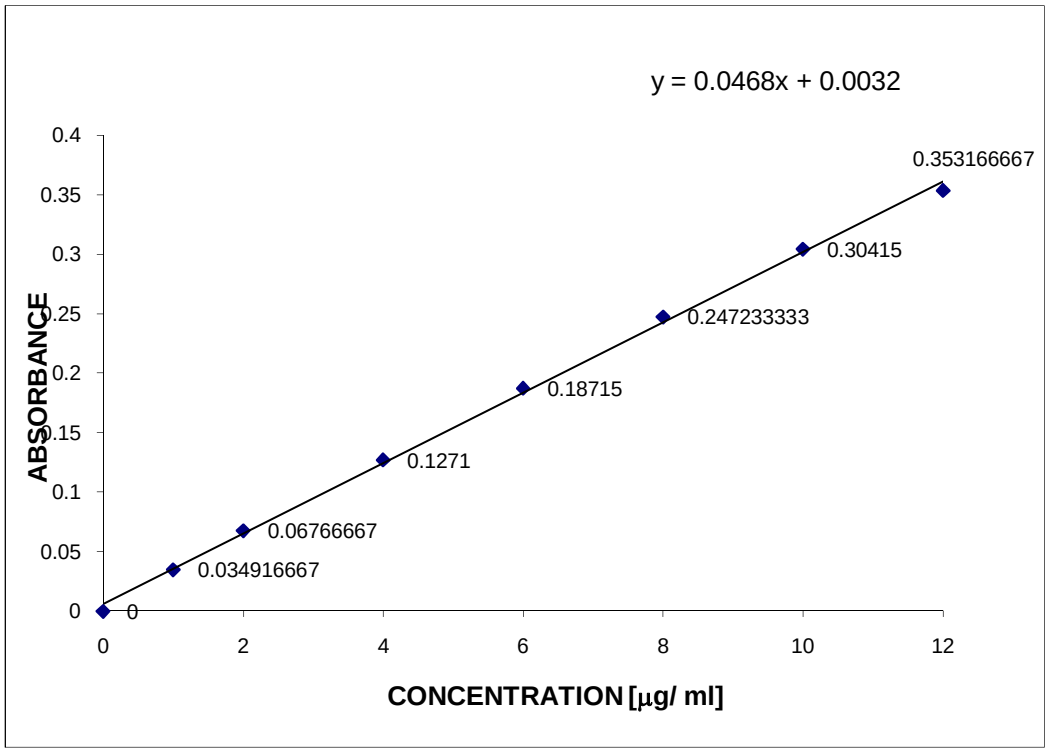


FIGURE – 6

CALIBRATION CURVE OF MONTELUKAST SODIUM

IN METHANOL AT 307 nm

(ABSORBANCE CORRECTION METHOD)

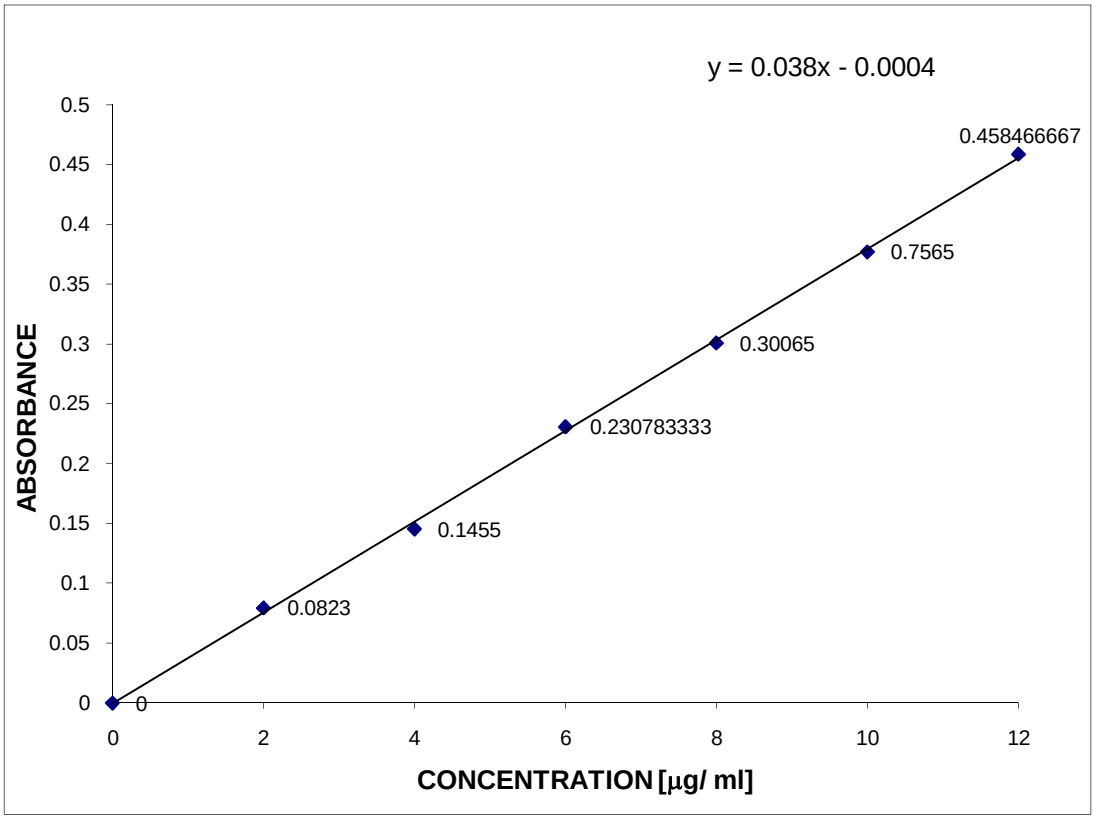


FIGURE – 7

CALIBRATION CURVE OF MONTELUKAST SODIUM

IN METHANOL AT 230 nm

(ABSORBANCE CORRECTION METHOD)

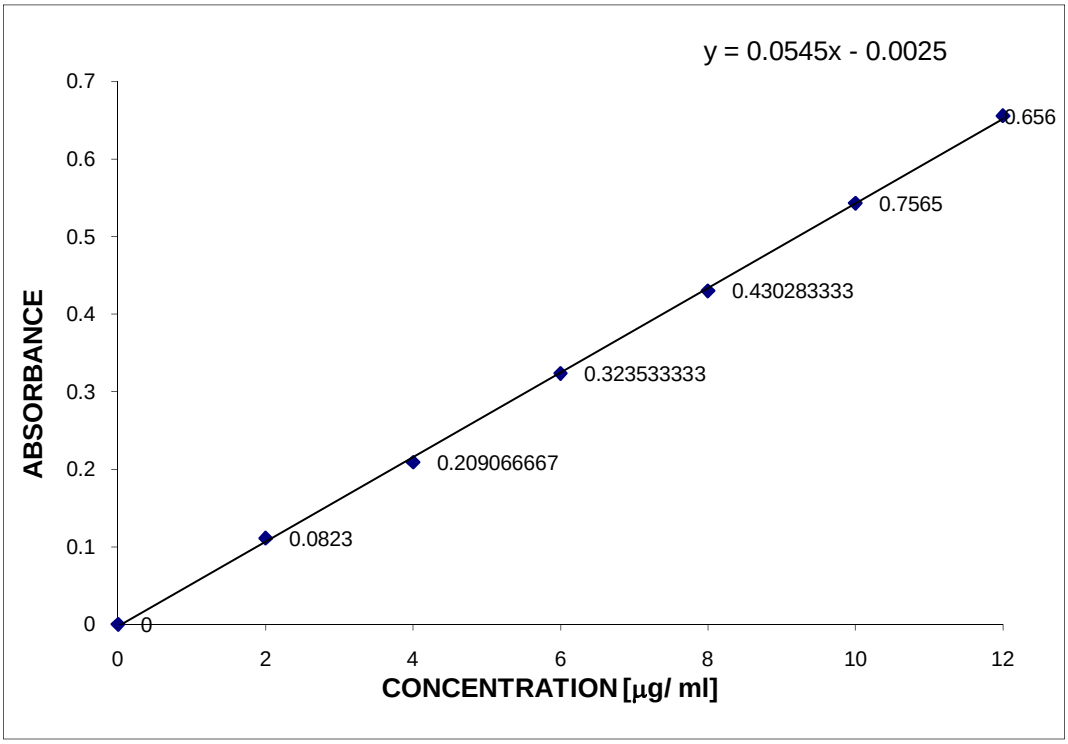


FIGURE – 8

CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE

IN METHANOL AT 307 nm

(ABSORBANCE CORRECTION METHOD)

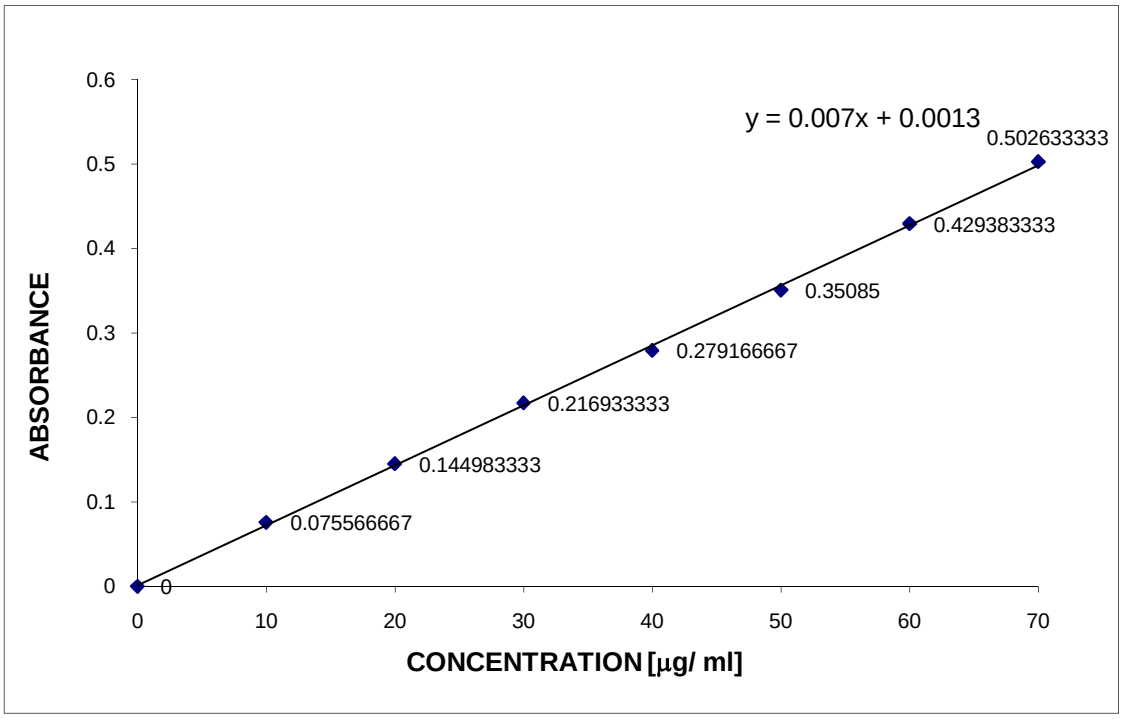


FIGURE – 9

CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE

IN METHANOL AT 230 nm

(ABSORBANCE CORRECTION METHOD)

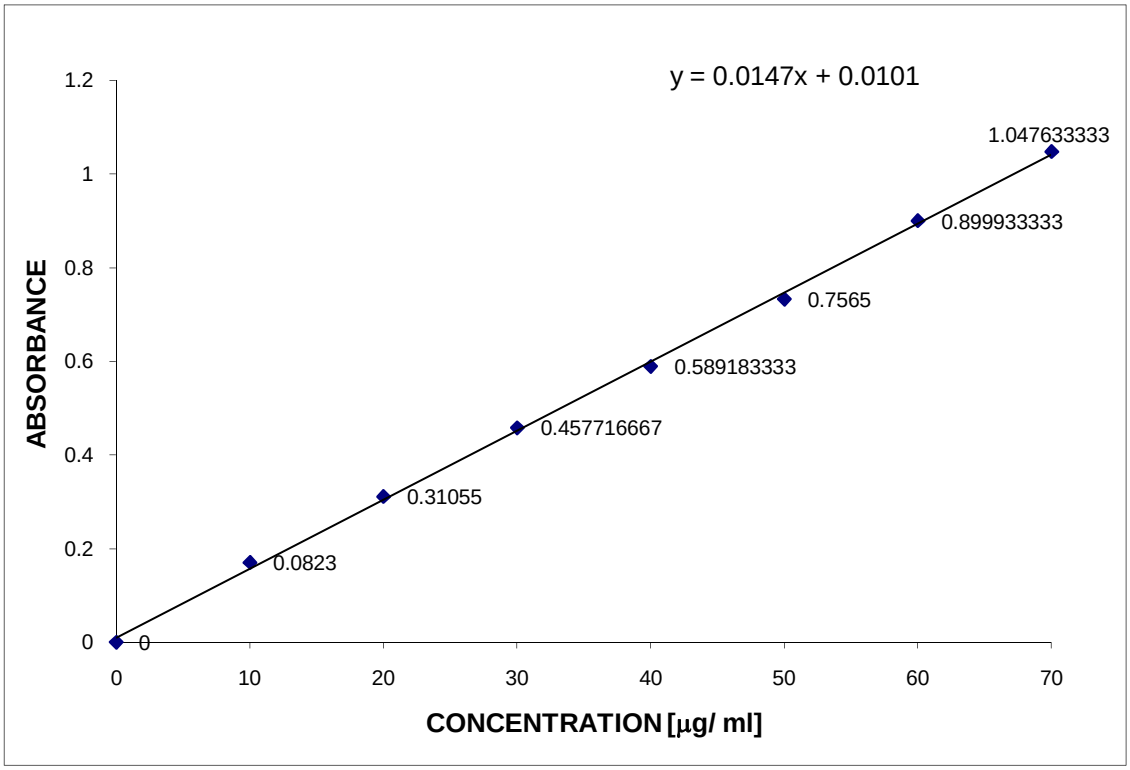


FIGURE – 10

CALIBRATION CURVE OF LEVOCETIRIZINE DIHYDROCHLORIDE

IN METHANOL AT 230 nm

(ABSORBANCE CORRECTION METHOD)

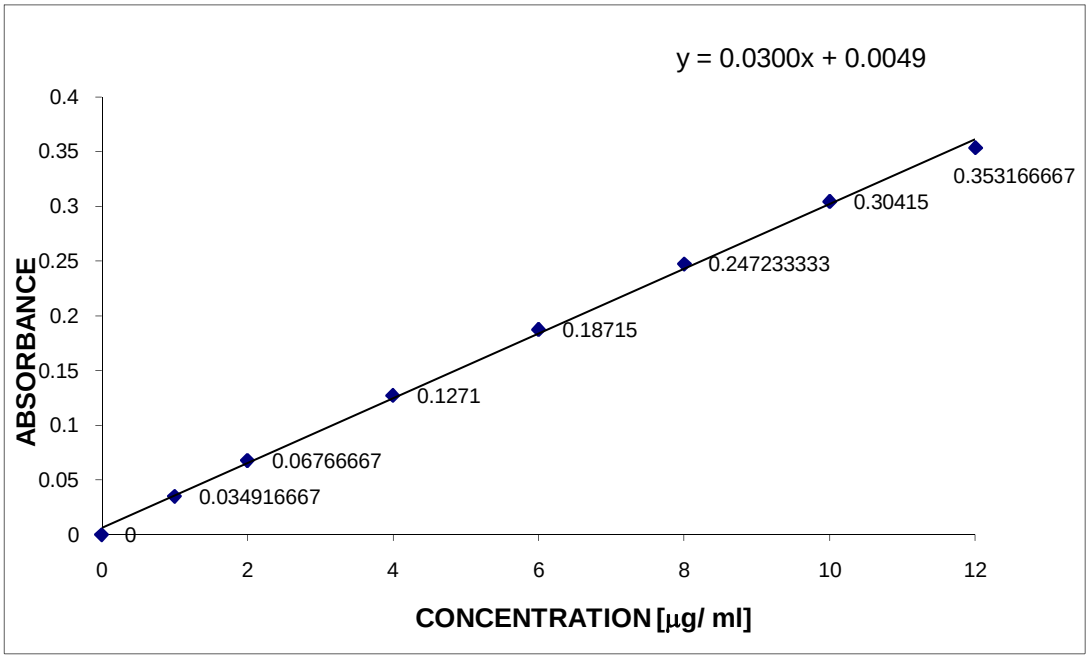
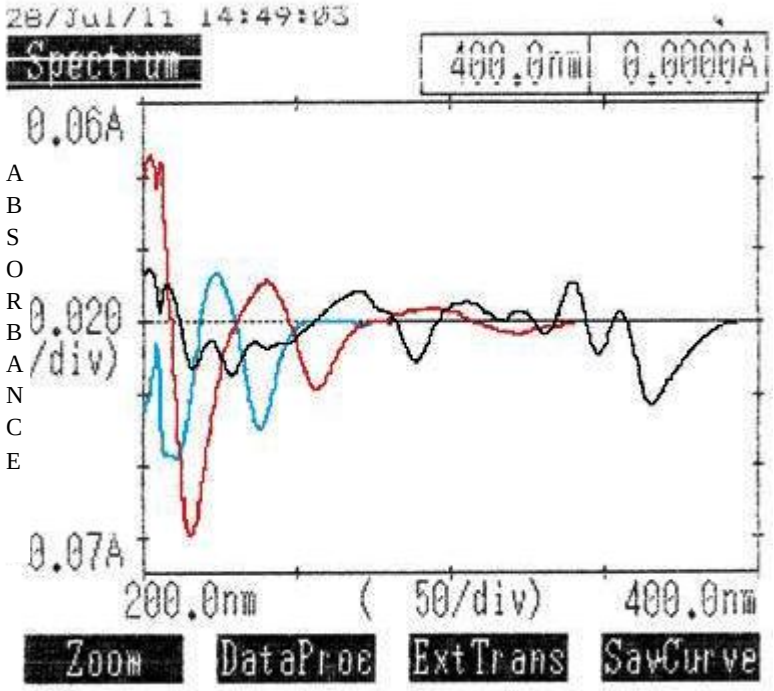


FIGURE - 11

UV OVERLAIN SPECTRUM OF MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE IN METHANOL (FIRST ORDER DERIVATIVE)



WAVELENGTH (nm)

- **Levocetirizine Dihydrochloride**
- **Ambroxol hydrochloride**
- **Montelukast Sodium**

FIGURE – 12

CALIBRATION CURVE OF MONTELUKAST SODIUM

IN METHANOL AT 365.5 nm

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

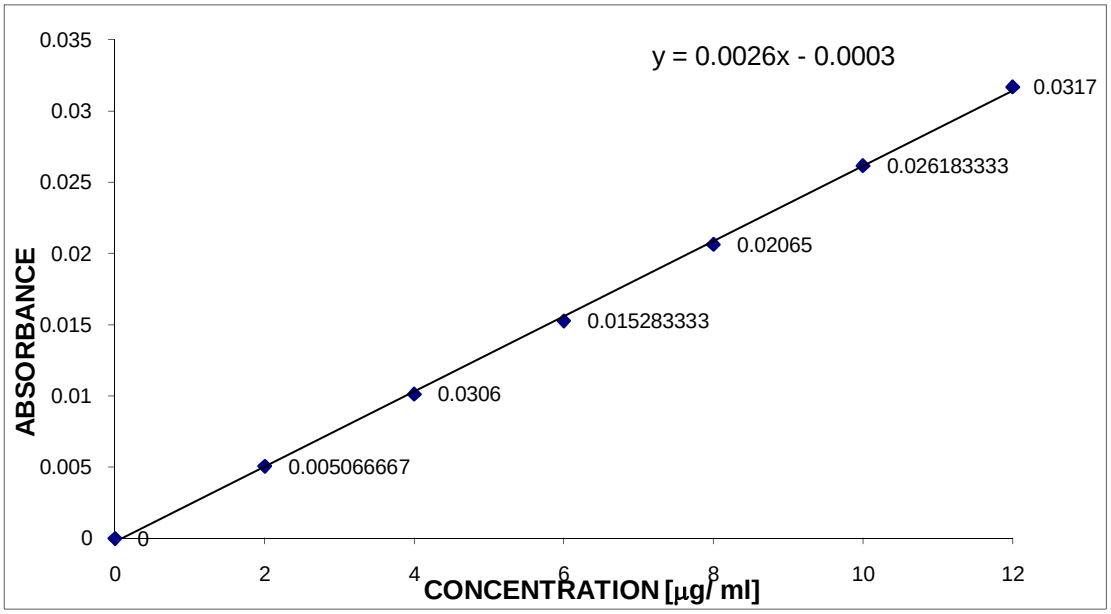


FIGURE – 13

CALIBRATION CURVE OF MONTELUKAST SODIUM

IN METHANOL AT 248 nm

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

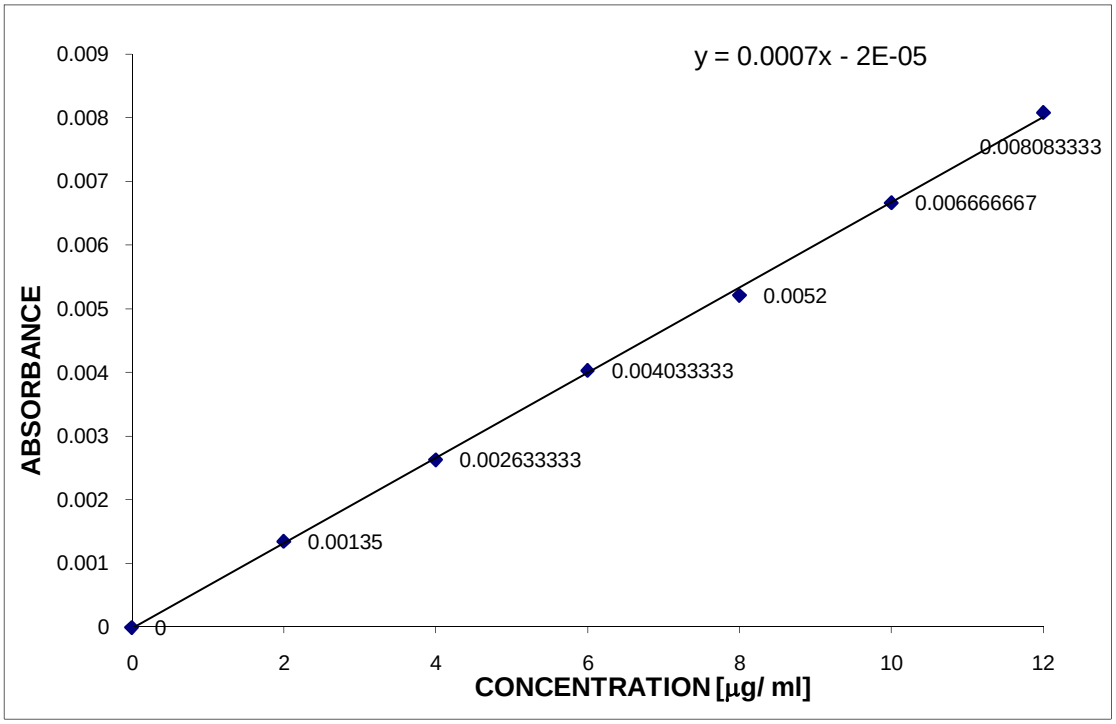


FIGURE – 14

CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE

IN METHANOL AT 256.5 nm

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

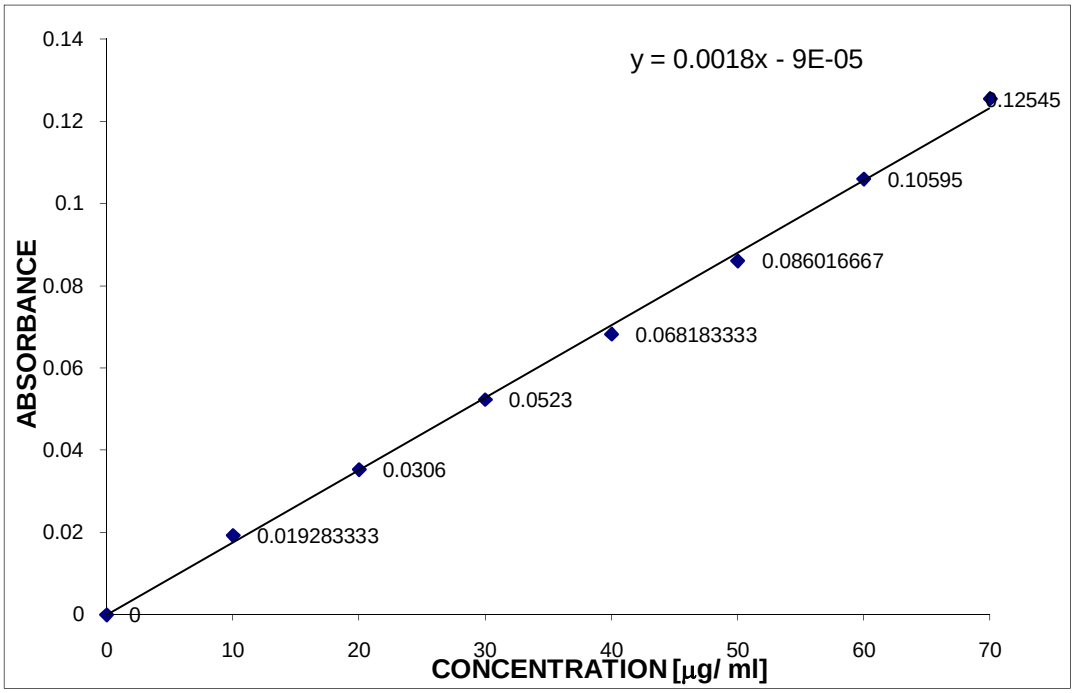


FIGURE – 15

CALIBRATION CURVE OF LEVOCETIRIZINE DIHYDROCHLORIDE

IN METHANOL AT 248 nm

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

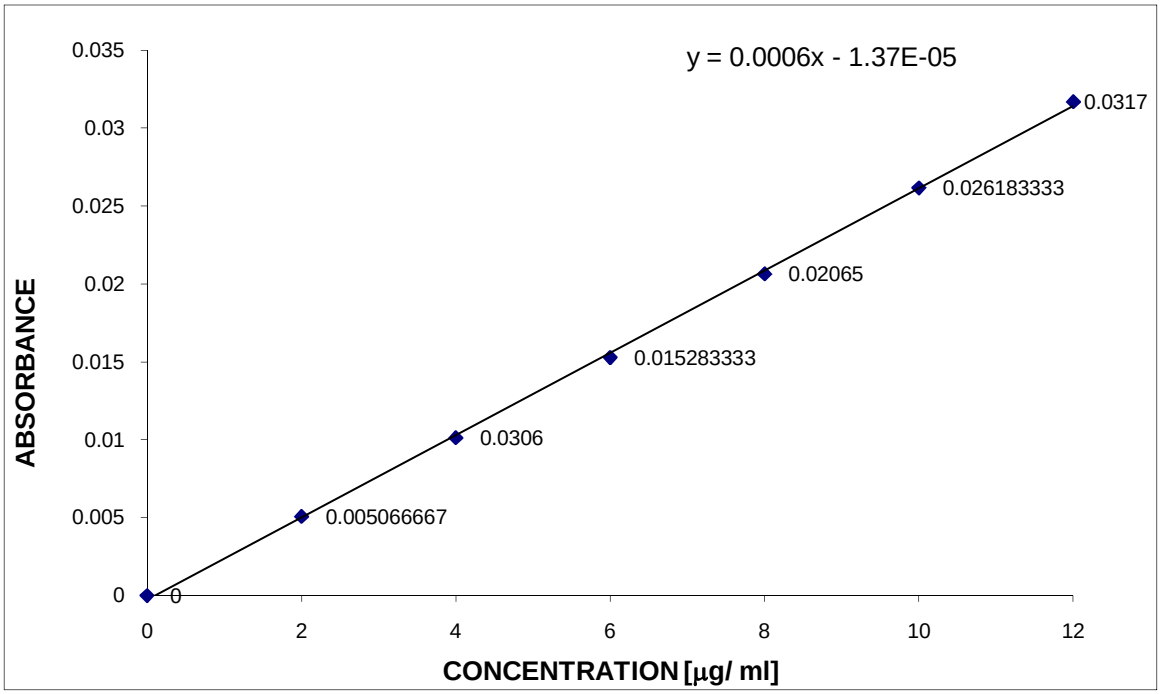
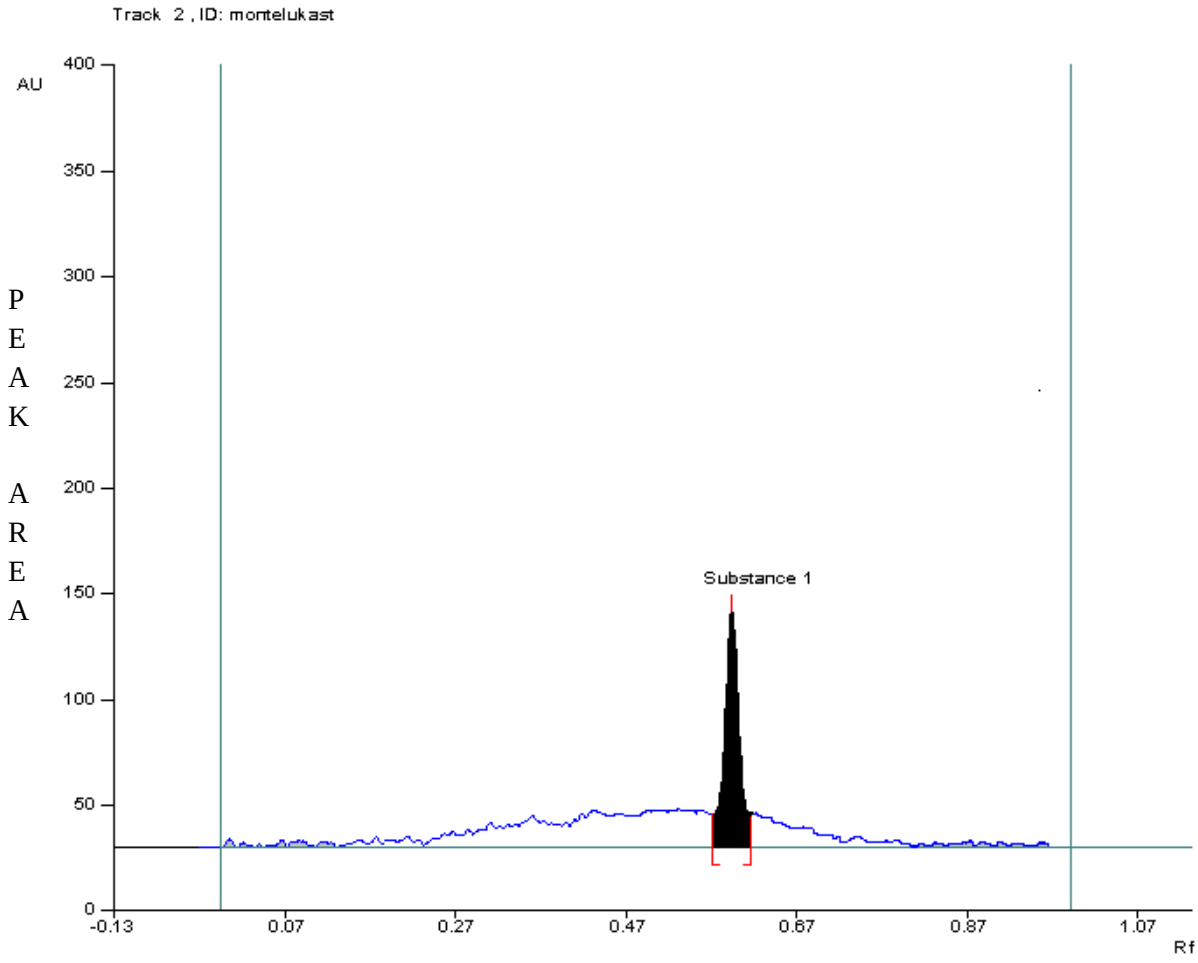


FIGURE – 16

OPTIMIZED CHROMATOGRAM FOR MONTELUKAST SODIUM (200 ng)

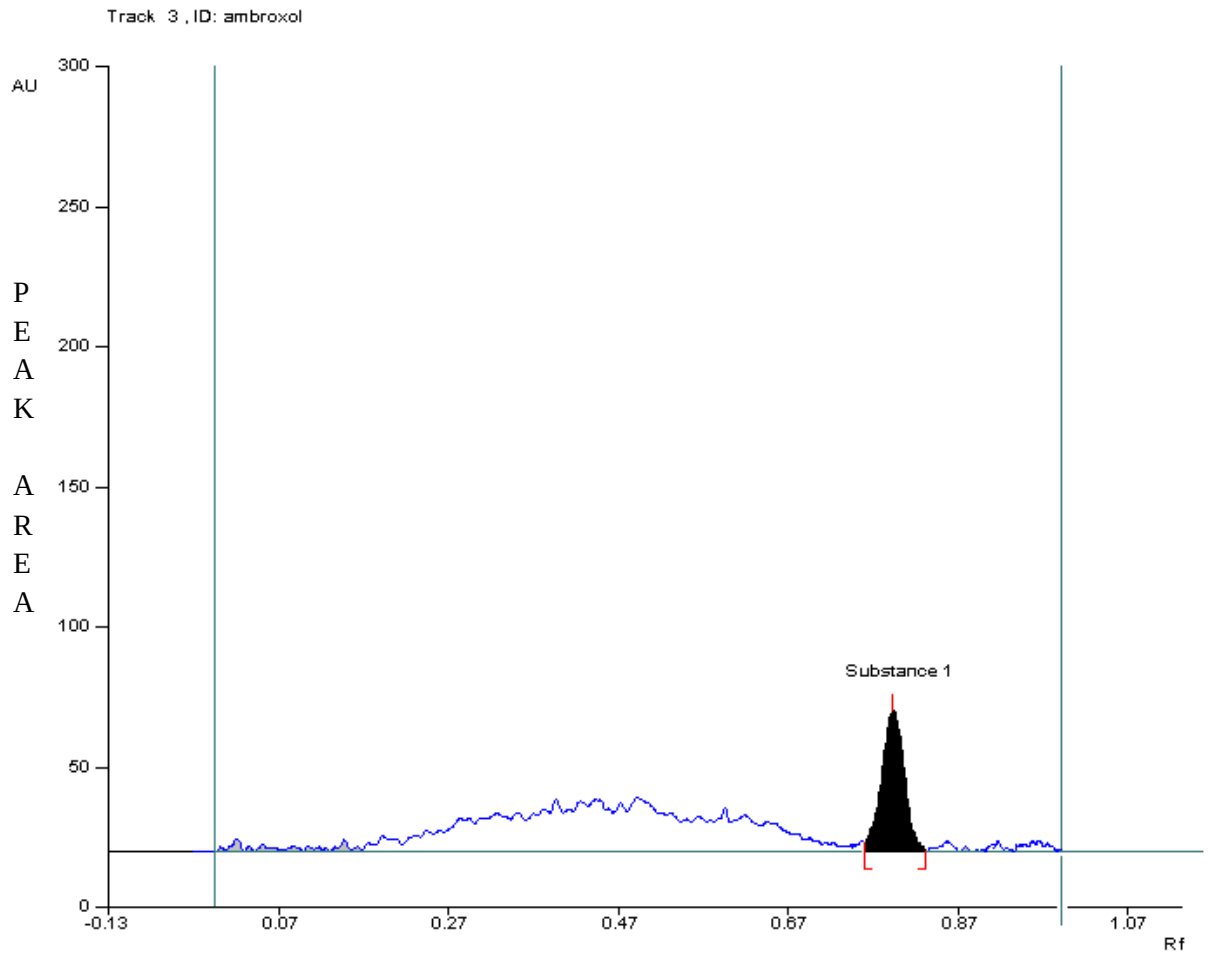


Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.56	1.3	0.58	90.61	100	0.60	1.1	3451.07	100	MON

FIGURE – 17

OPTIMIZED CHROMATOGRAM FOR AMBROXOL HYDROCHLORIDE

(200 ng)

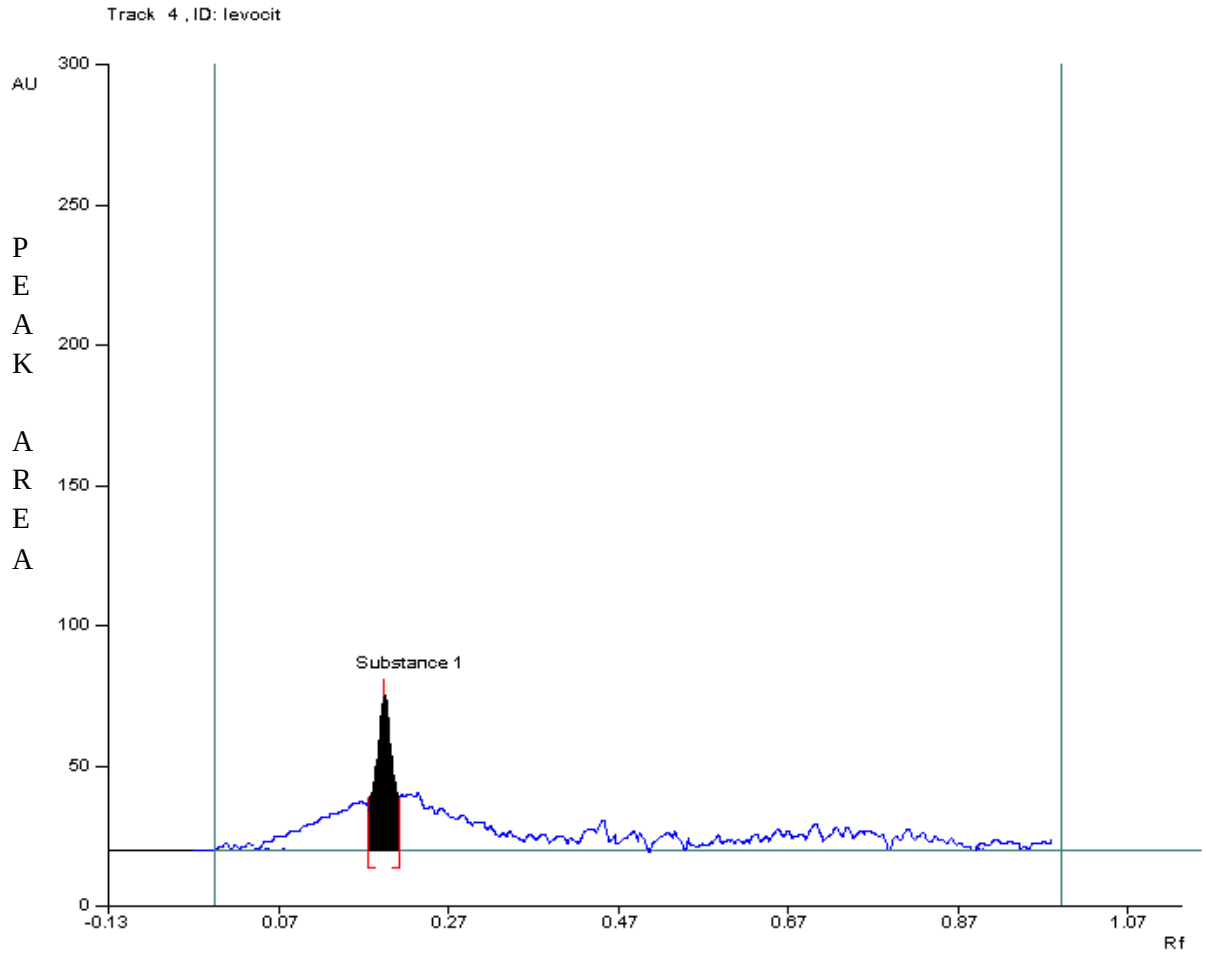


RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.74	1.3	0.76	34.6	25.77	0.80	1.2	941.4	100	AMB

FIGURE – 18

OPTIMIZED CHROMATOGRAM FOR LEVOCETIRIZINE DIHYDROCHLORIDE (200 ng)



RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	1.3	0.20	44.6	45.77	0.22	1.6	2253.21	100	LEVO

FIGURE – 19

OVERLAIN UV – SPECTRUM OF MONTELUKAST SODIUM, AMBROXOL HYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE FOR THE SELECTION OF DETECTION WAVELENGTH

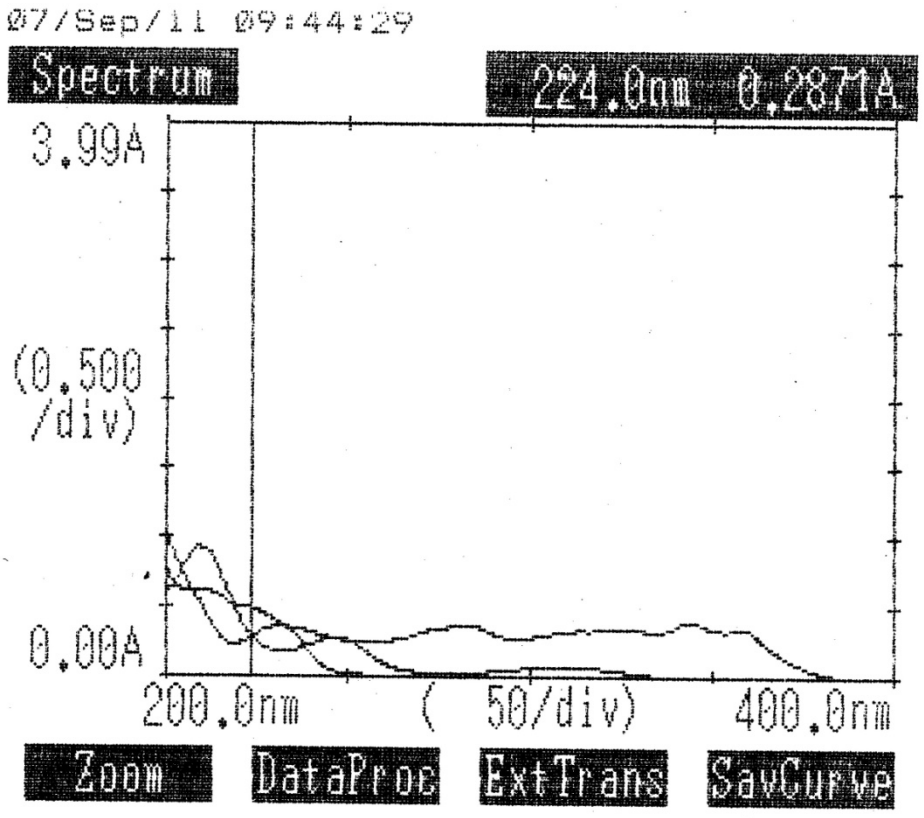
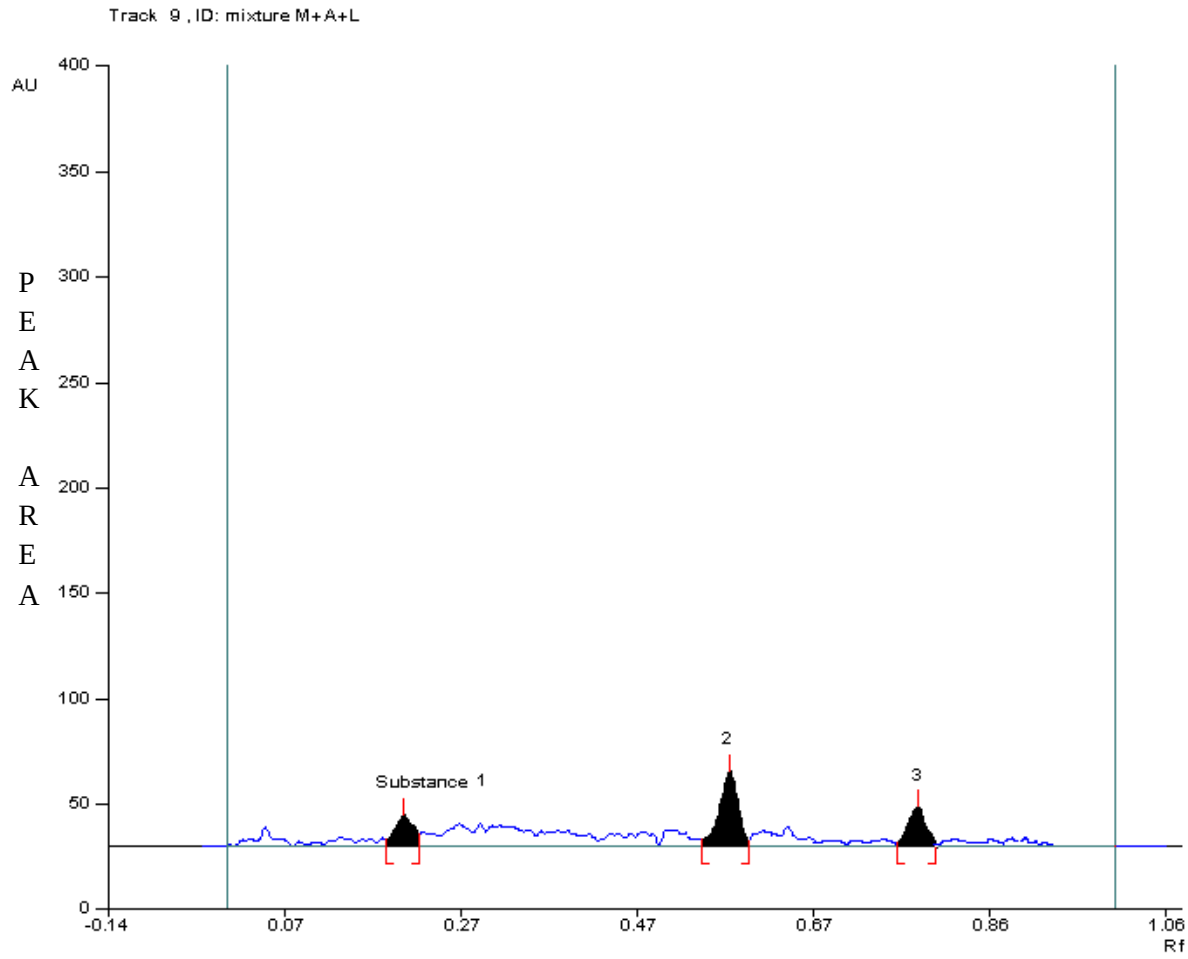


FIGURE - 20

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM,

LEVOCETIRIZINE DIHYDROCHLORIDE AND

AMBROXOL HYDROCHLORIDE (20, 20, 75 ng)

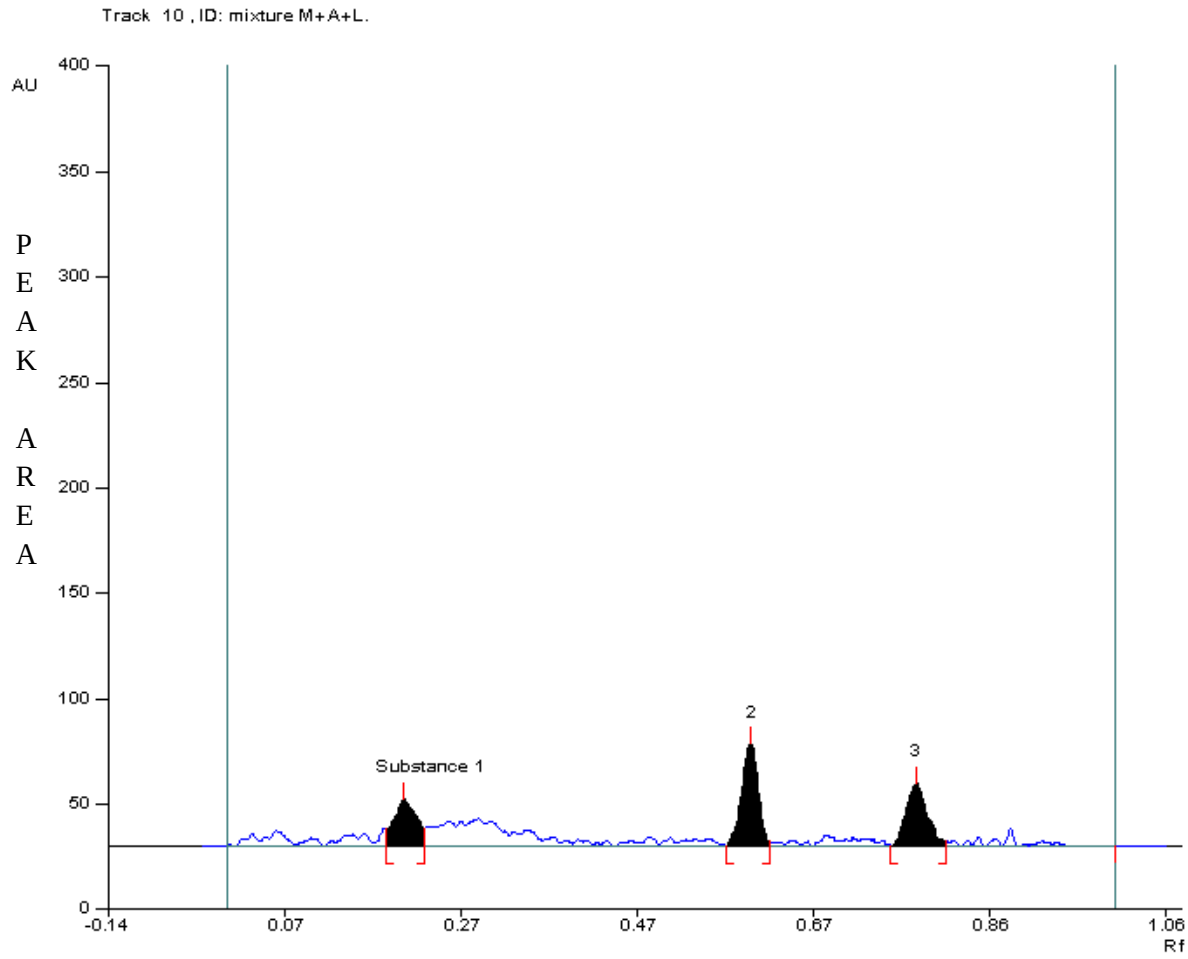


RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.2	10.6	25.77	0.22	3.6	257.2	25.24	LEVO
2.	0.56	2.6	0.59	32.1	44.01	0.61	1.9	431.32	37.98	MON
3.	0.76	1.4	0.78	18.9	37.36	0.79	0.9	336.2	36.32	AMB

FIGURE – 21

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE (40, 40, 150 ng)

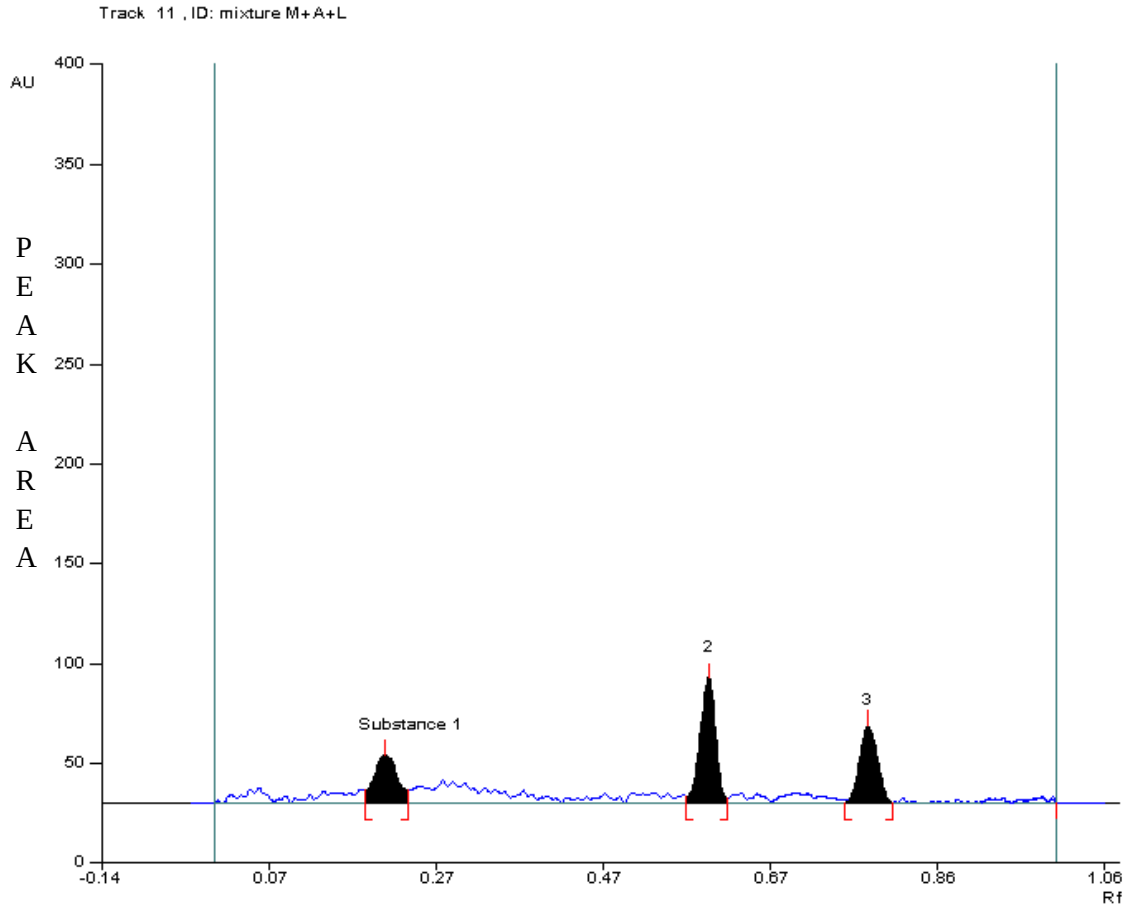


RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	13.6	25.77	0.23	3.6	501.32	25.24	LEVO
2.	0.56	2.6	0.59	52.1	44.01	0.61	1.9	789.87	37.98	MON
3.	0.76	1.4	0.78	28.9	37.36	0.79	0.9	643.22	36.32	AMB

FIGURE – 22

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE (60, 60, 225 ng)

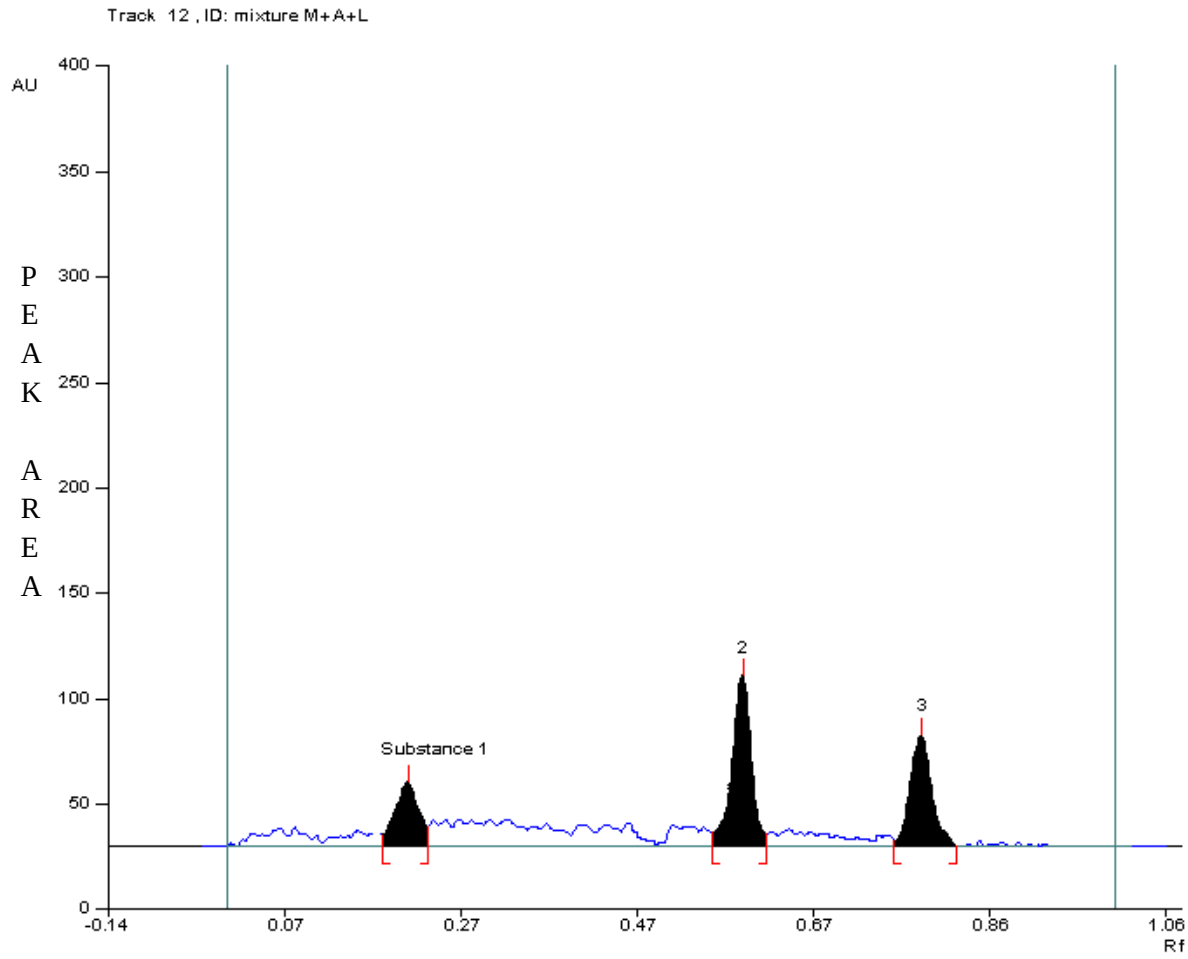


RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	15.6	25.77	0.22	3.6	764.09	27.95	LEVO
2.	0.56	2.6	0.59	62.1	44.01	0.61	1.9	1123.3	37.61	MON
3.	0.76	1.4	0.78	36.8	37.36	0.79	0.9	954.21	34.38	AMB

FIGURE – 23

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE (80, 80, 300 ng)



RETENTION FACTOR

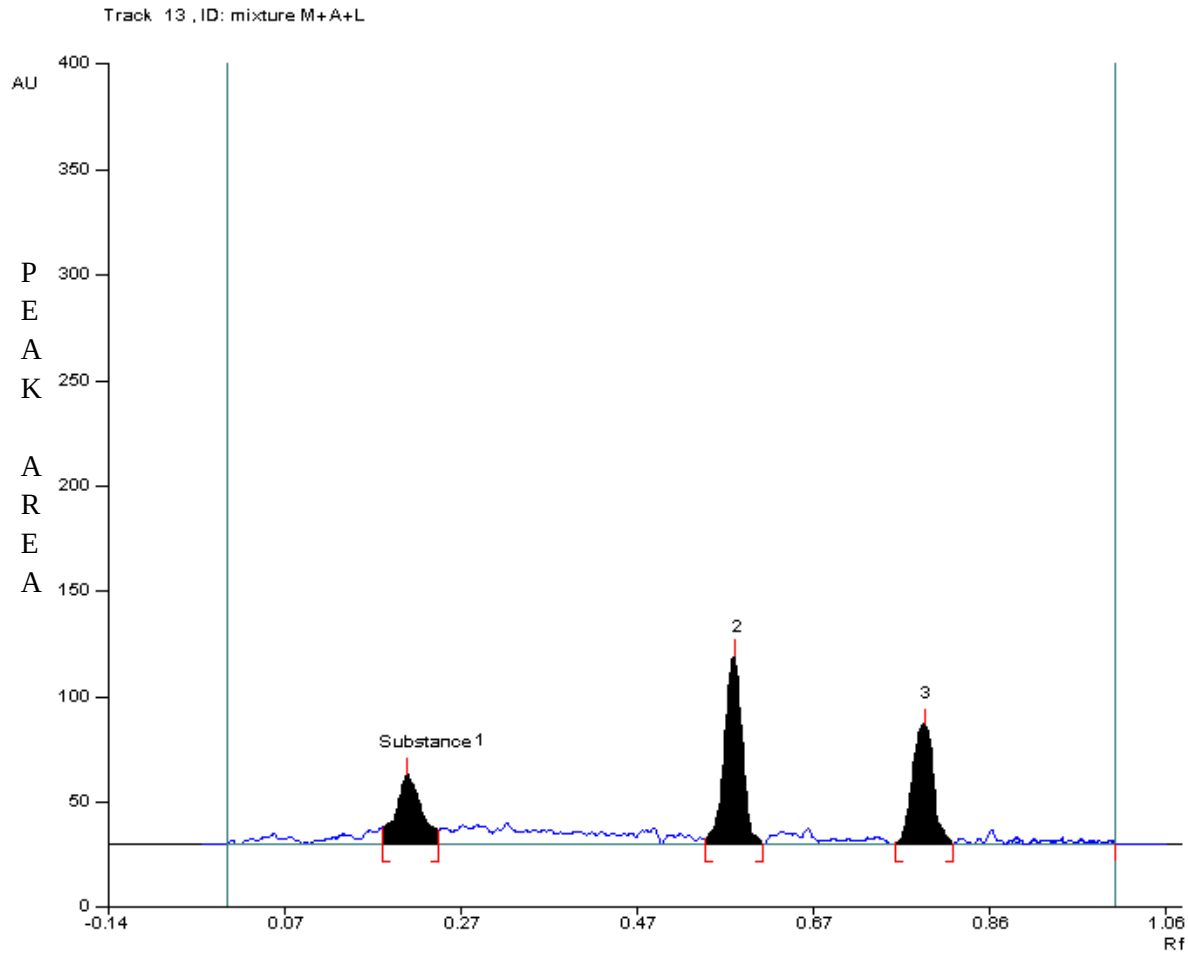
Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.2	20.7	25.77	0.22	3.6	1032.32	28.99	LEVO
2.	0.56	2.6	0.59	72.8	44.01	0.61	1.9	1468.32	36.41	MON
3.	0.74	1.4	0.79	38.6	37.36	0.81	0.9	1291.32	34.59	AMB

FIGURE – 24

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM,

LEVOCETIRIZINE DIHYDROCHLORIDE AND

AMBROXOL HYDROCHLORIDE (100, 100, 375 ng)



RETENTION FACTOR

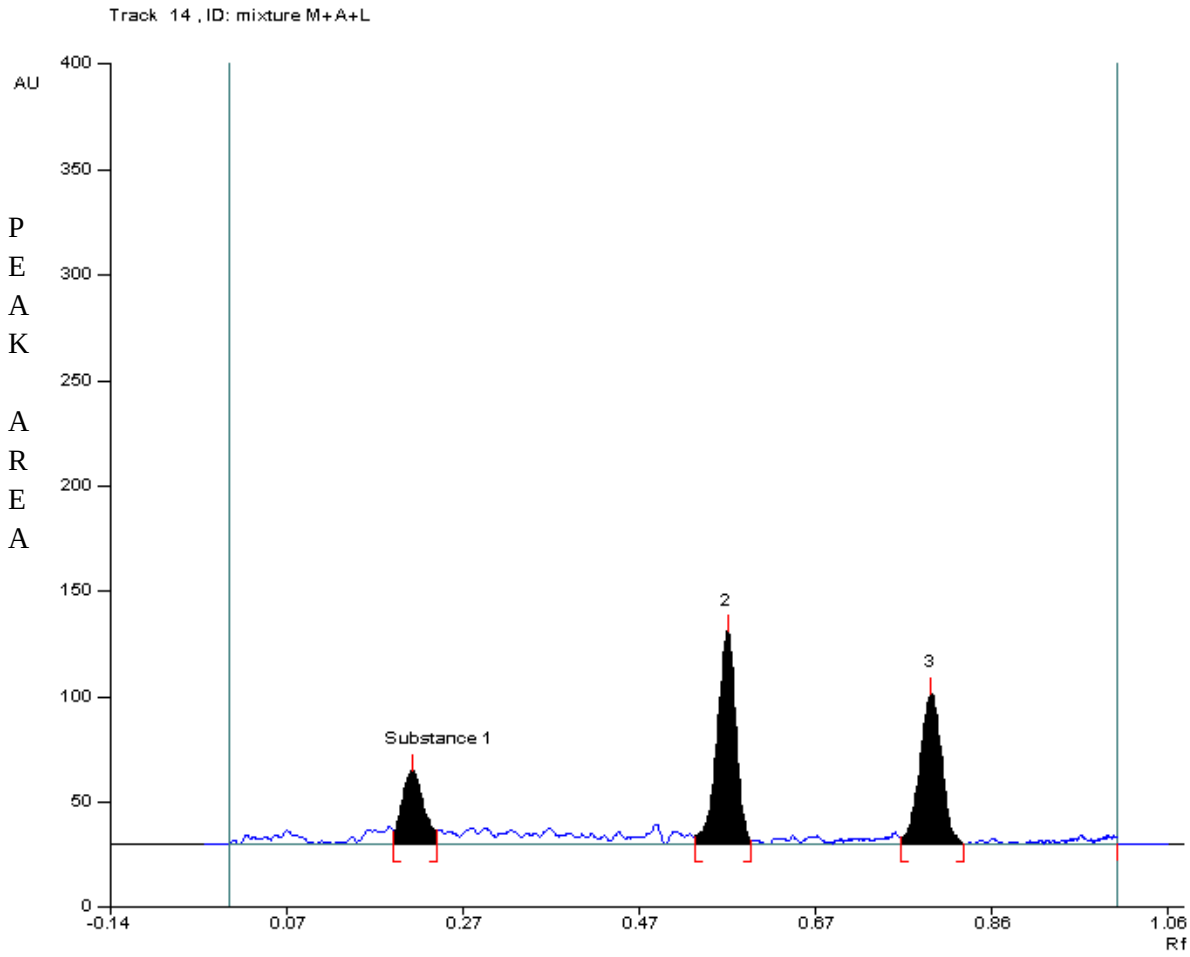
Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	24.6	25.77	0.22	3.6	1277.34	26.98	LEVO
2.	0.56	2.6	0.59	82.1	44.01	0.61	1.9	1837.98	36.64	MON
3.	0.76	1.4	0.78	46.9	37.36	0.79	0.9	1588.98	33.42	AMB

FIGURE – 25

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM,

LEVOCETIRIZINE DIHYDROCHLORIDE AND

AMBROXOL HYDROCHLORIDE (120, 120, 450 ng)



RETENTION FACTOR

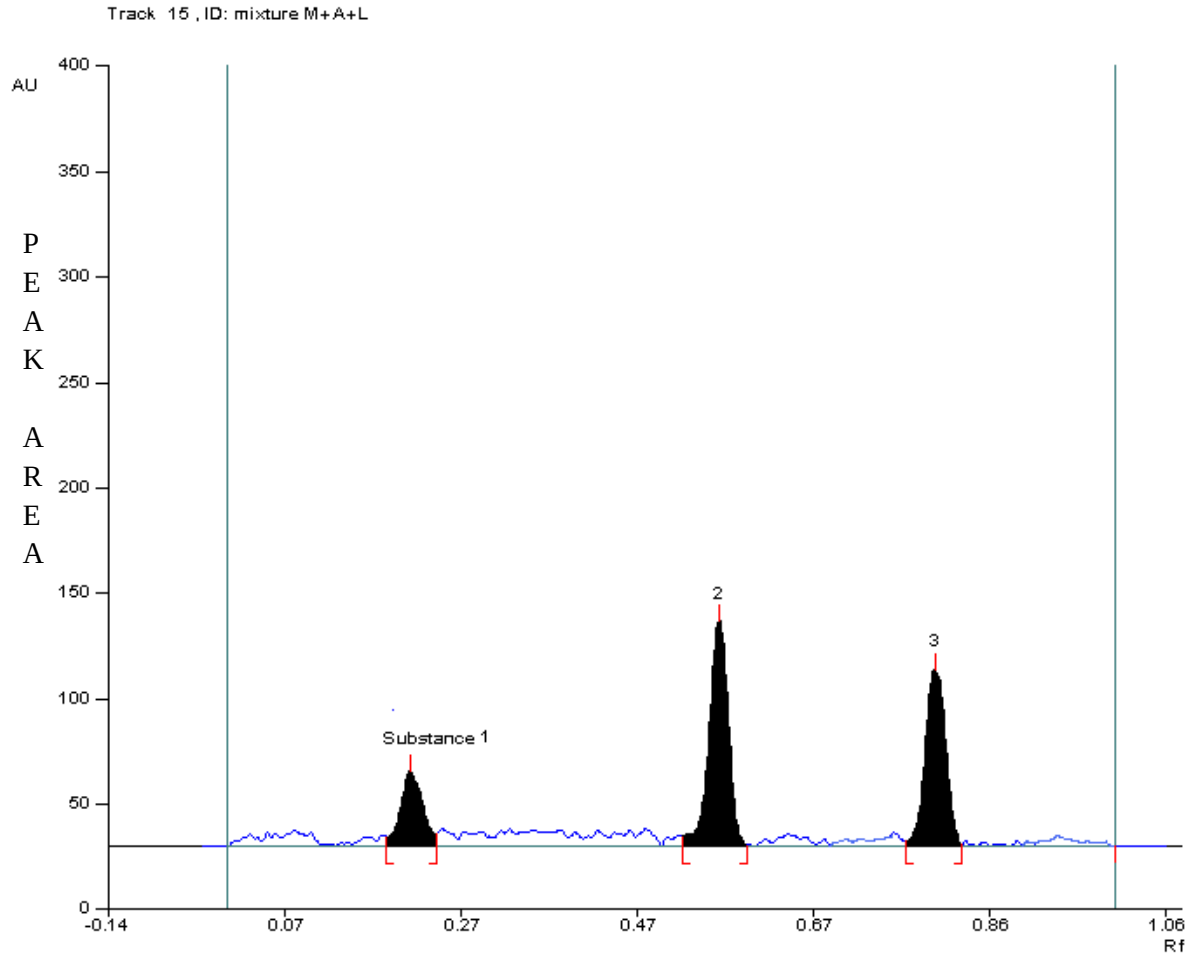
Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.2	28.1	25.77	0.22	3.6	1576.32	30.16	LEVO
2.	0.56	2.6	0.59	83.1	44.01	0.60	1.9	2178.65	37.40	MON
3.	0.76	1.4	0.78	58.4	37.36	0.80	0.9	1821.22	34.21	AMB

FIGURE – 26

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM,

LEVOCETIRIZINE DIHYDROCHLORIDE AND

AMBROXOL HYDROCHLORIDE (140, 140, 525 ng)



RETENTION FACTOR

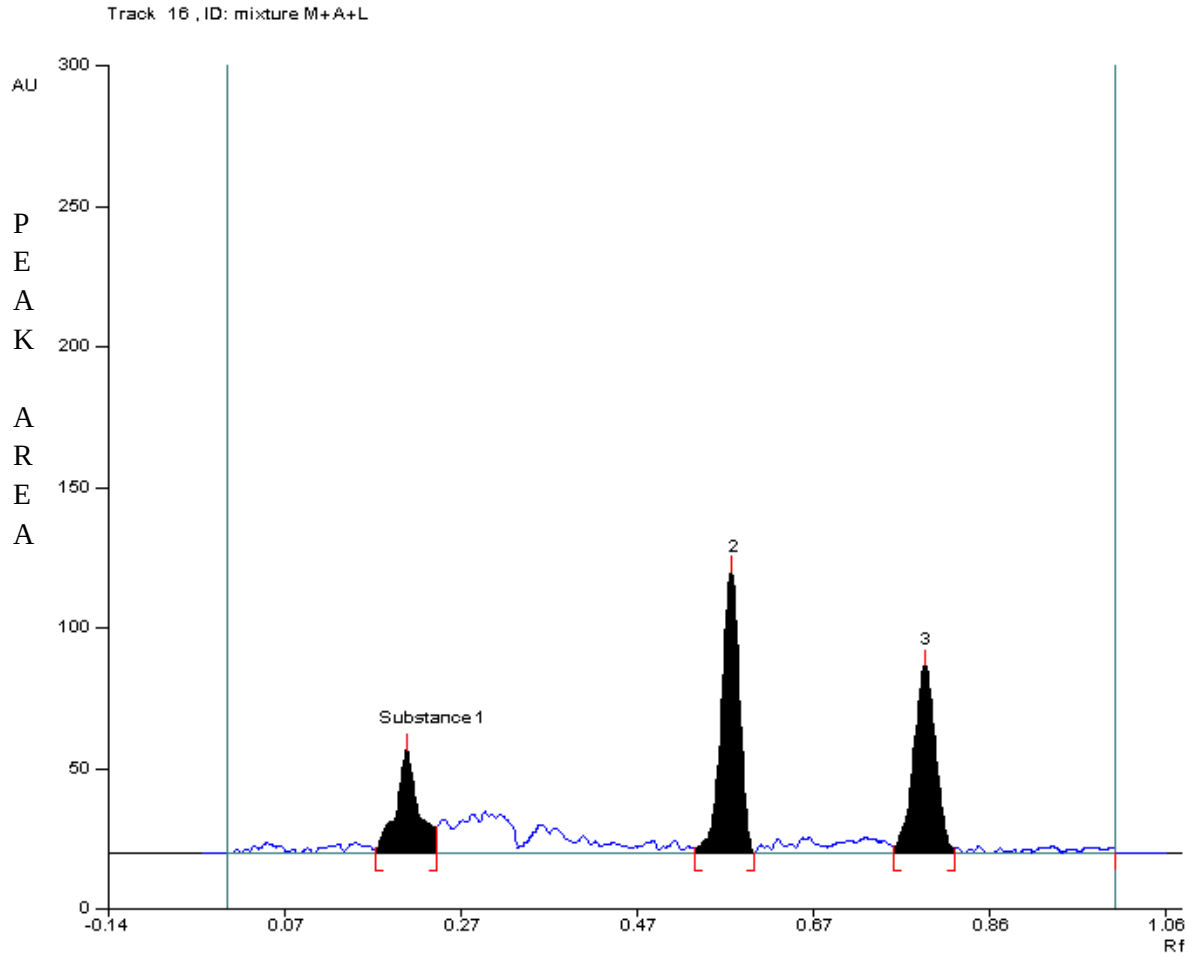
Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	37.6	25.77	0.23	3.6	1785.32	27.98	LEVO
2.	0.56	2.6	0.59	102.1	44.01	0.62	1.9	2578.65	36.31	MON
3.	0.76	1.4	0.78	67.5	37.36	0.81	0.9	2178.23	34.12	AMB

FIGURE – 27

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM,

LEVOCETIRIZINE DIHYDROCHLORIDE AND

AMBROXOL HYDROCHLORIDE (160, 160, 600 ng)



RETENTION FACTOR

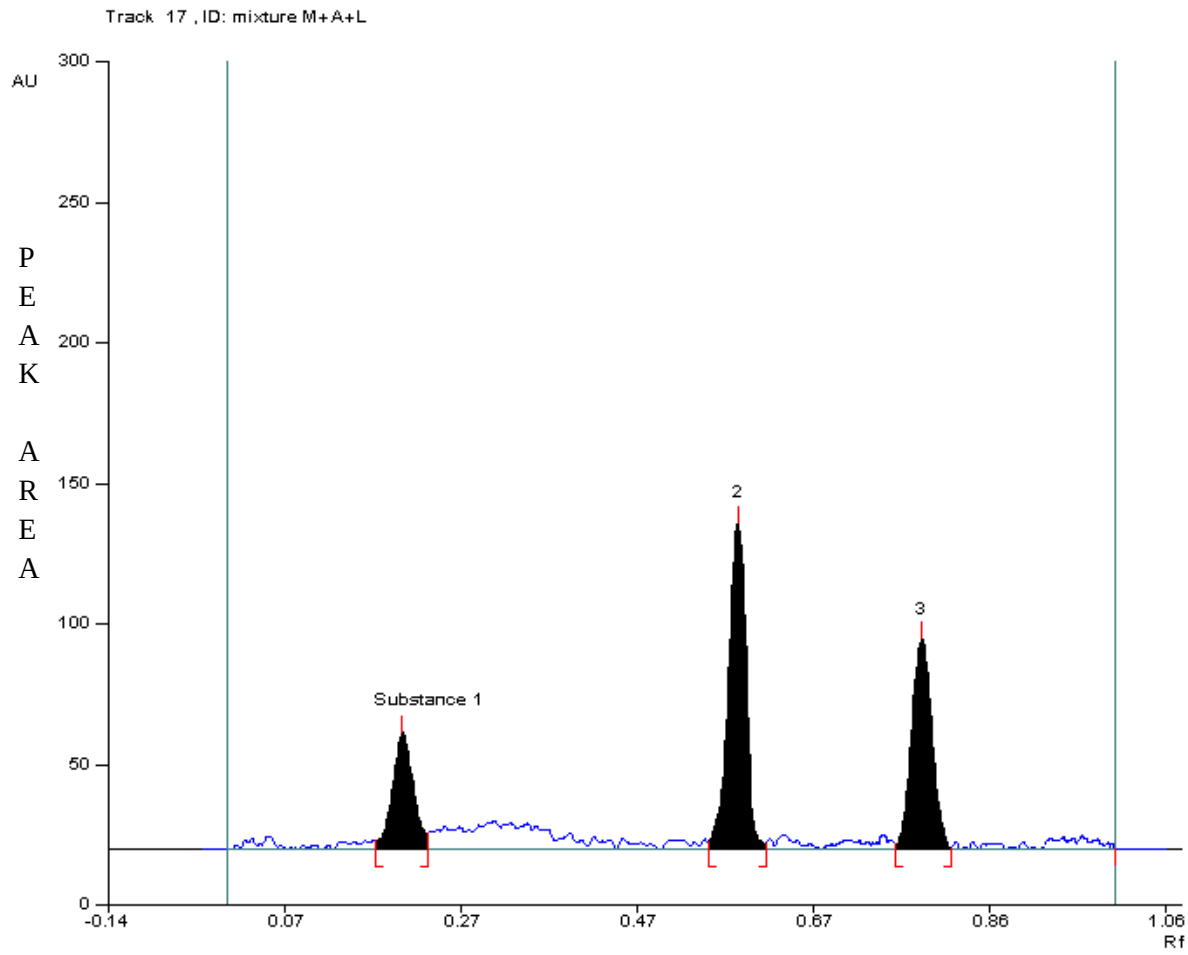
Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.2	41.1	25.77	0.22	3.6	2043.56	26.54	LEVO
2.	0.56	2.6	0.59	112.1	44.01	0.61	1.9	2888.21	35.43	MON
3.	0.76	1.4	0.78	78.1	37.36	0.79	0.9	2598.23	35.61	AMB

FIGURE – 28

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM,

LEVOCETIRIZINE DIHYDROCHLORIDE AND

AMBROXOL HYDROCHLORIDE (180, 180, 675 ng)



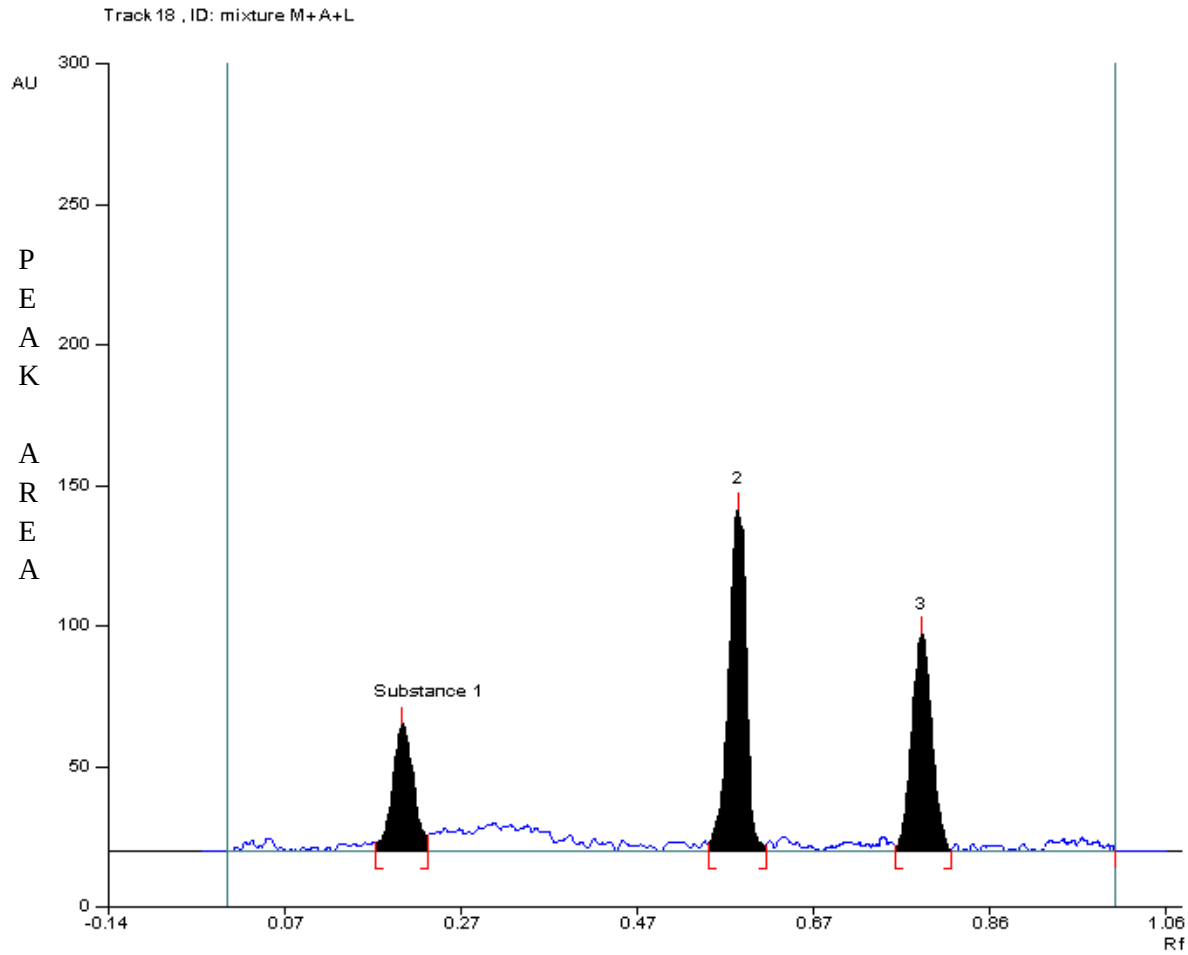
Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.20	47.6	25.77	0.23	3.6	2221.10	27.34	LEVO
2.	0.56	2.6	0.59	129.1	44.01	0.63	1.9	3266.87	33.76	MON
3.	0.76	1.4	0.79	88.9	37.36	0.81	0.9	2799.87	32.43	AMB

FIGURE – 29

LINEARITY CHROMATOGRAM OFMONTELUKAST SODIUM,

LEVOCETIRIZINE DIHYDROCHLORIDE AND

AMBROXOL HYDROCHLORIDE (200, 200, 750 ng)



Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.20	54.6	25.77	0.22	3.6	2634.33	23.89	LEVO
2.	0.56	2.6	0.59	132.1	44.01	0.61	1.9	3451.87	33.10	MON
3.	0.76	1.4	0.78	91.7	37.36	0.79	0.9	3197.34	33.21	AMB

FIGURE – 30

CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE

BY HPTLC

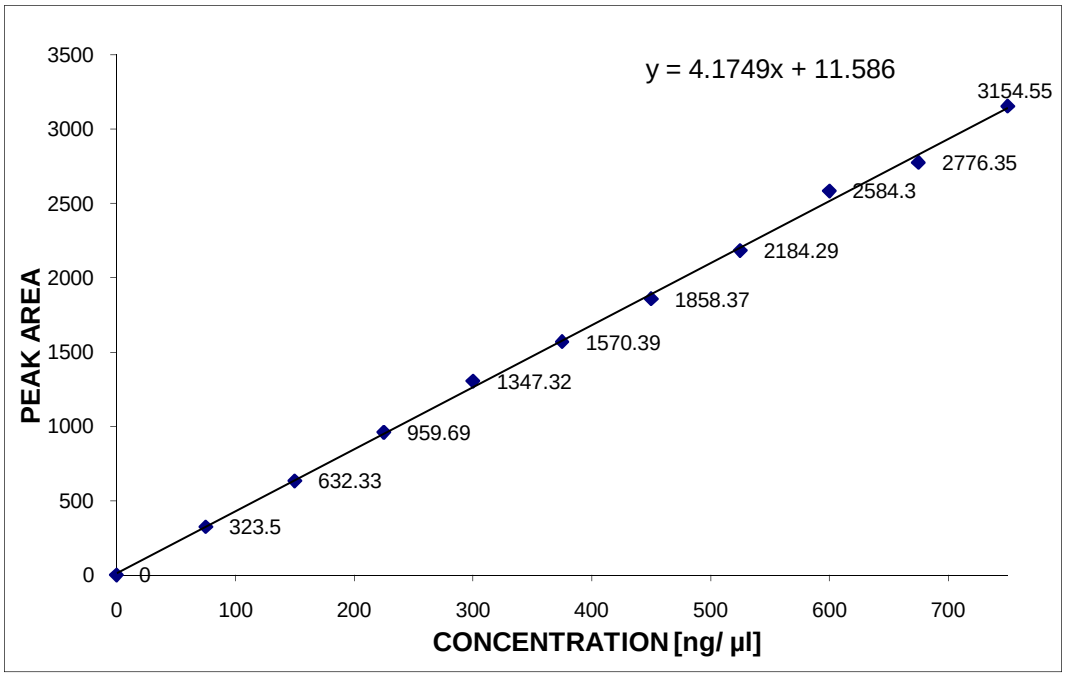


FIGURE – 31

CALIBRATION CURVE OF LEVOCETIRIZINE DIHYDROCHLORIDE

BY HPTLC

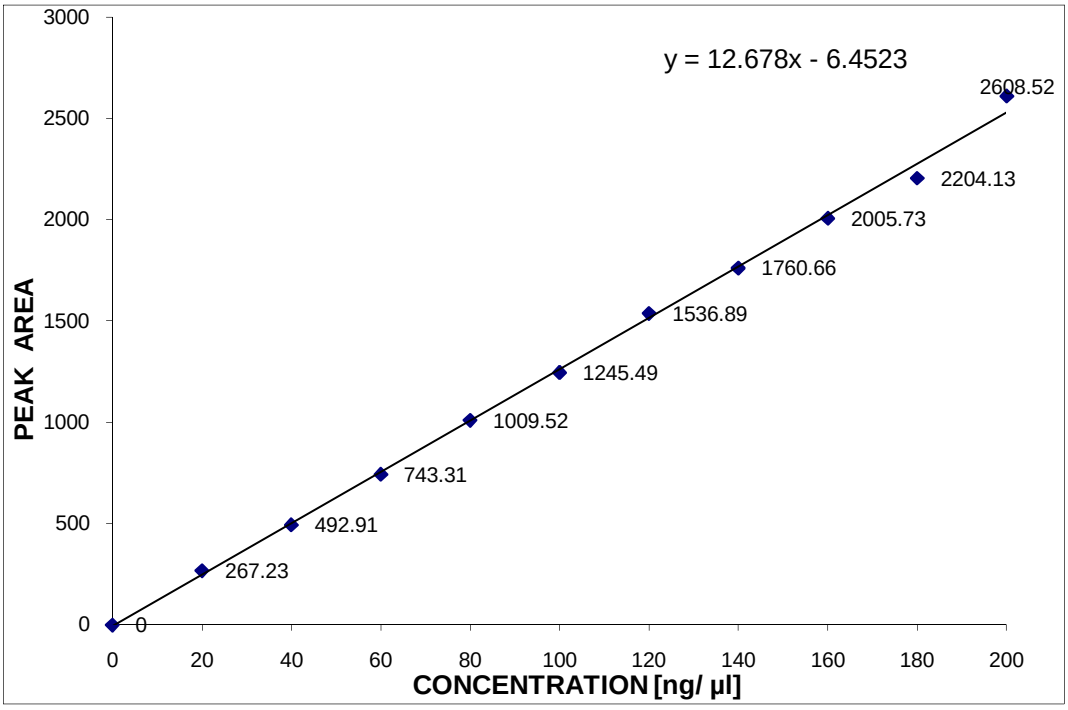


FIGURE – 32

CALIBRATION CURVE OF MONTELUKAST SODIUM BY HPTLC

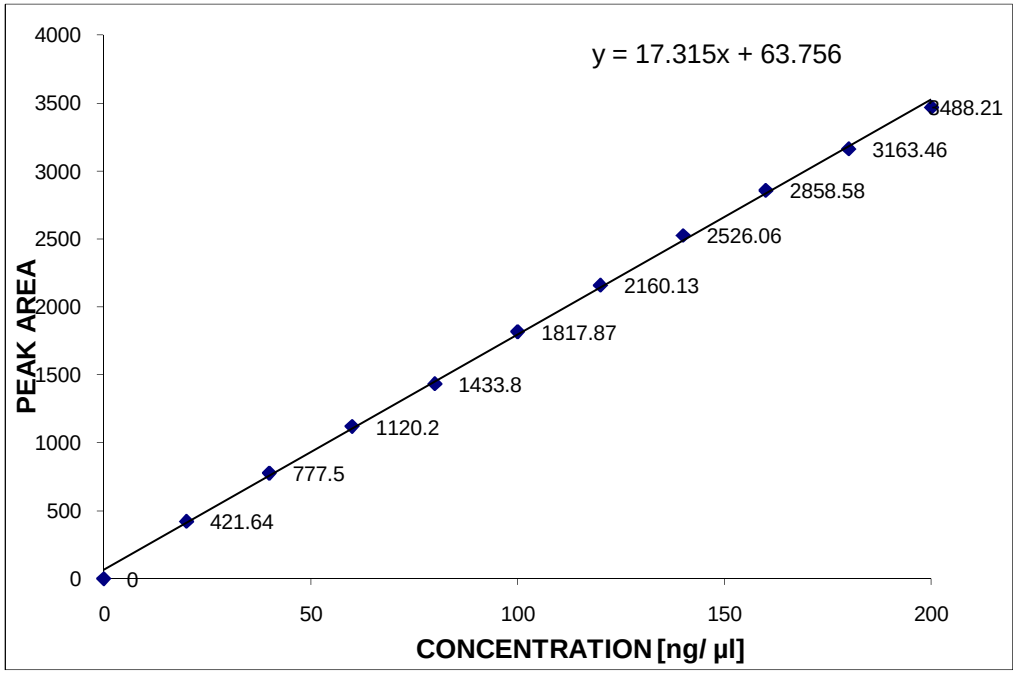
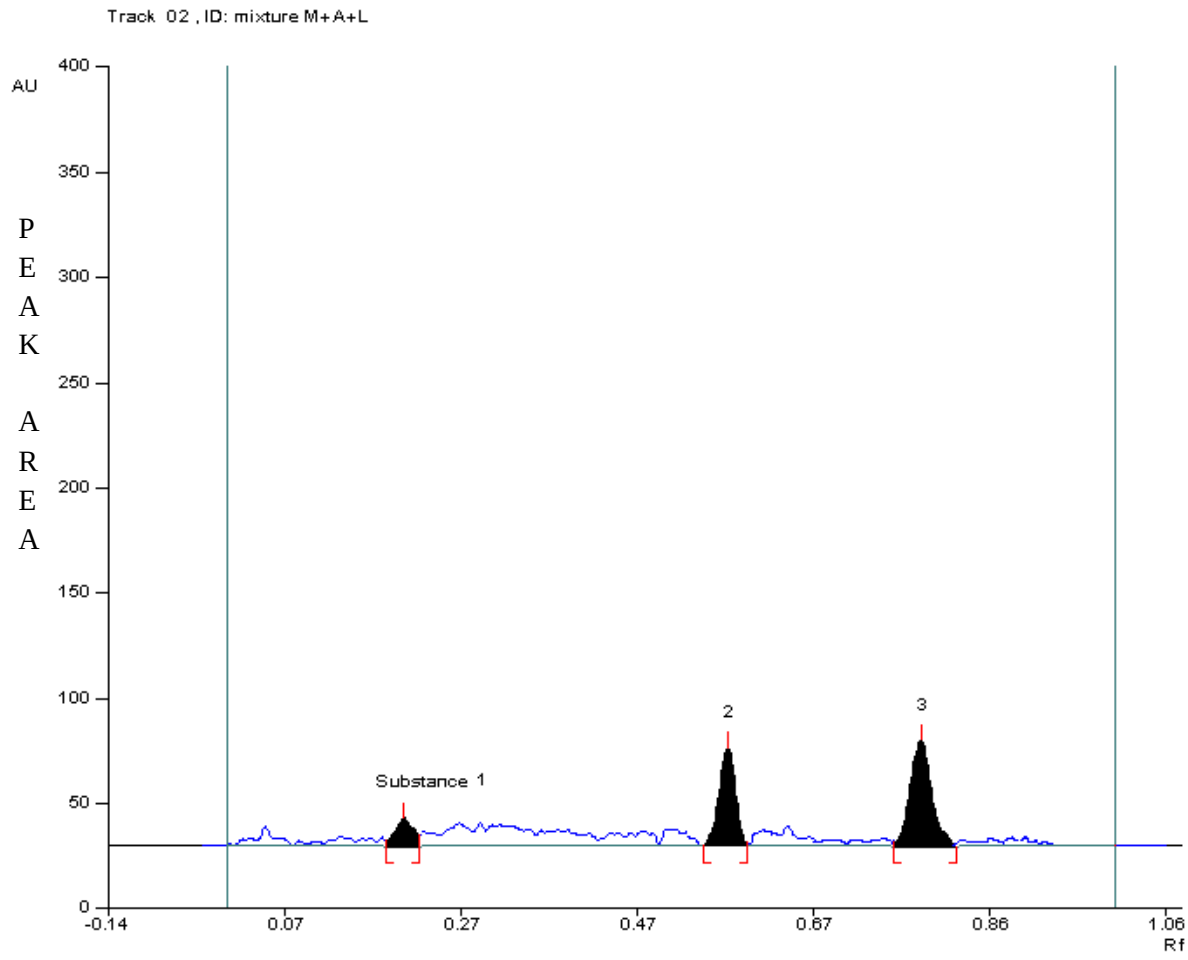


FIGURE – 33

ANALYSIS OF FORMULATION – RENEA

REPEATABILITY - 1



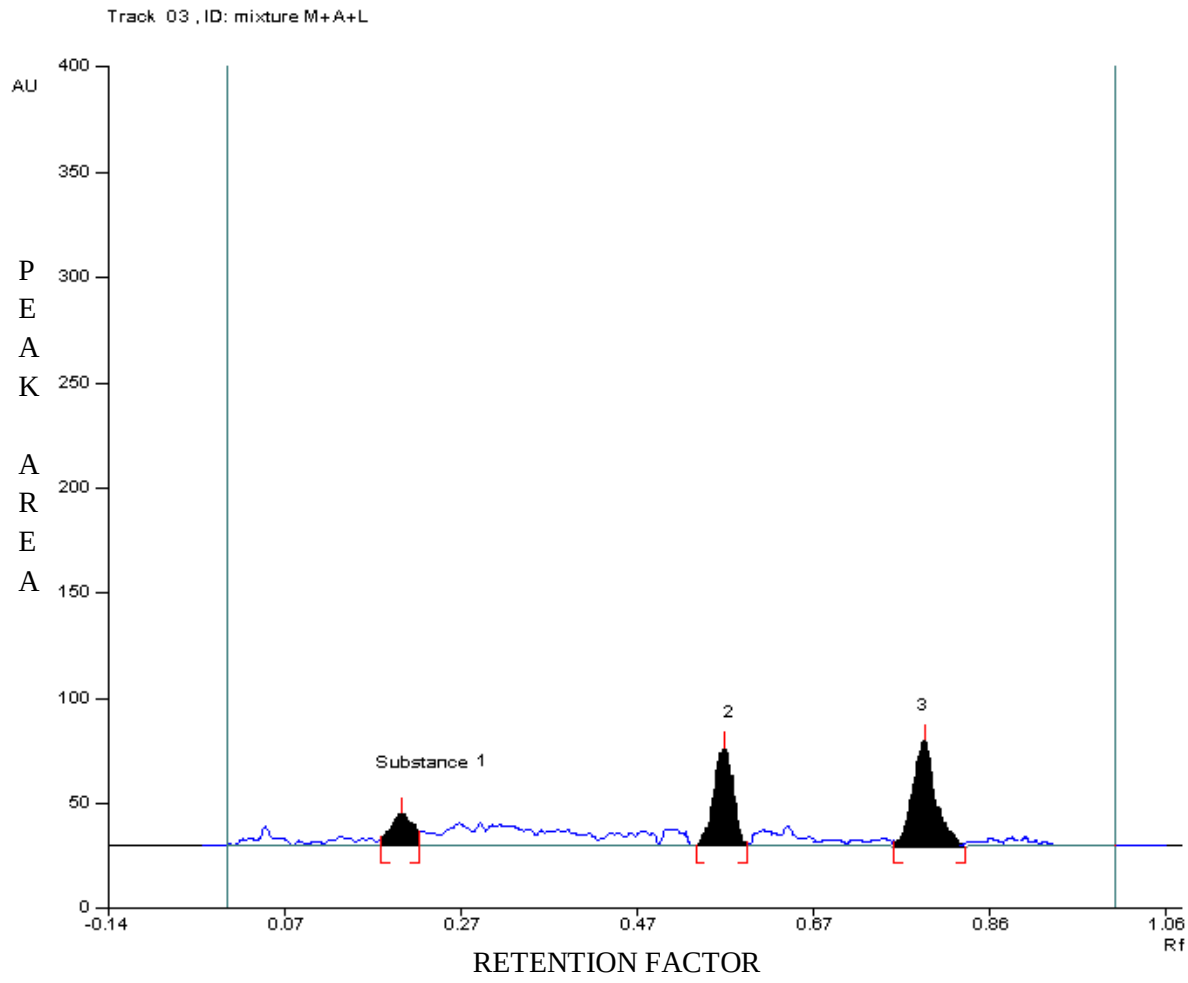
RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	15.6	25.77	0.22	3.1	251.32	18.87	LEVO
2.	0.56	2.6	0.59	33.1	44.01	0.62	0.67	772.7	32.21	MON
3.	0.76	1.4	0.78	47.6	37.36	0.79	0.5	1298.9	51.21	AMB

FIGURE – 34

ANALYSIS OF FORMULATION - RENEA

REPEATABILITY – 2

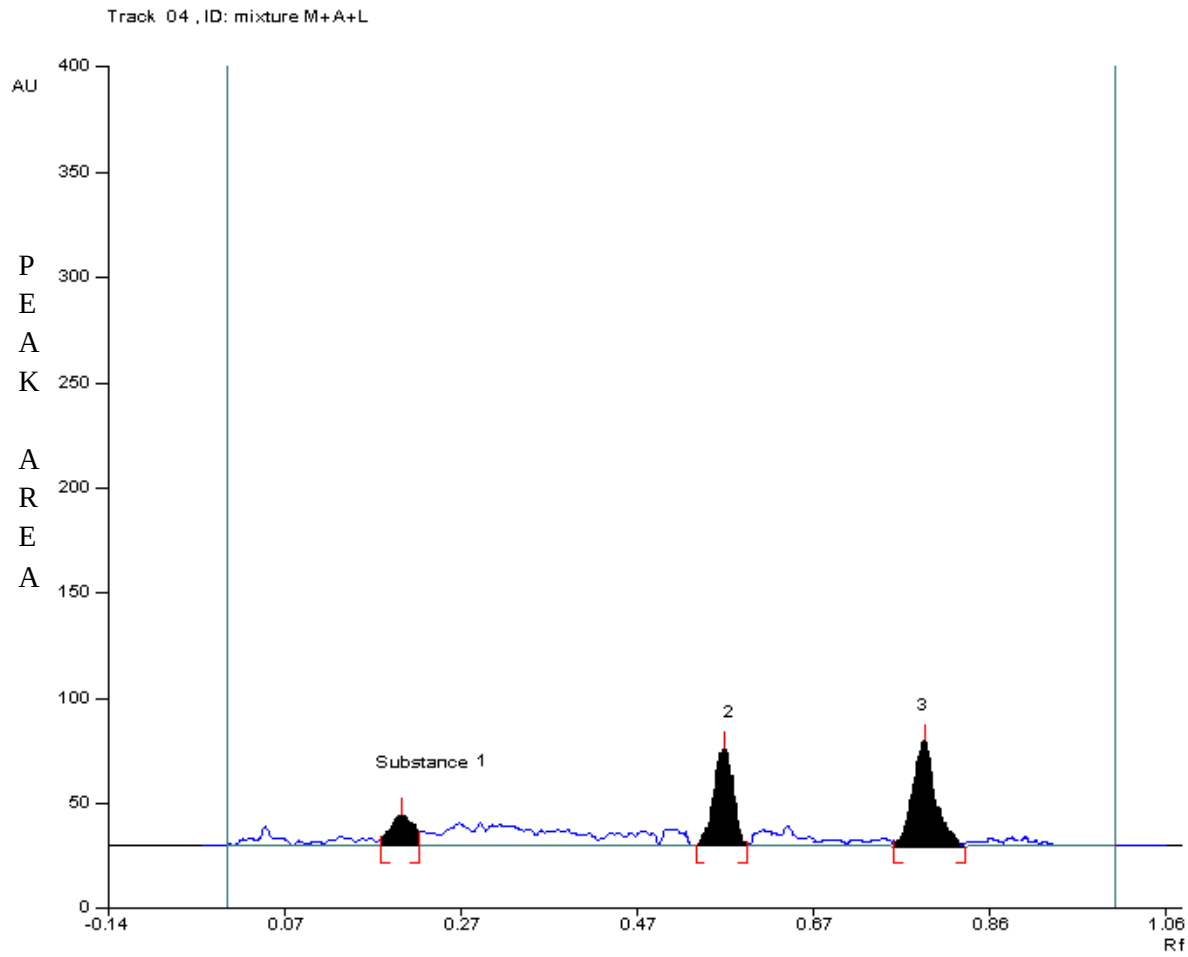


Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.20	15.3	25.77	0.22	1.2	253.3	25.24	LEVO
2.	0.56	2.6	0.59	32.1	44.01	0.61	0.7	776.7	31.4	MON
3.	0.76	1.4	0.78	48.1	37.36	0.79	0.2	1291.9	51.23	AMB

FIGURE – 35

ANALYSIS OF FORMULATION – RENEA

REPEATABILITY - 3



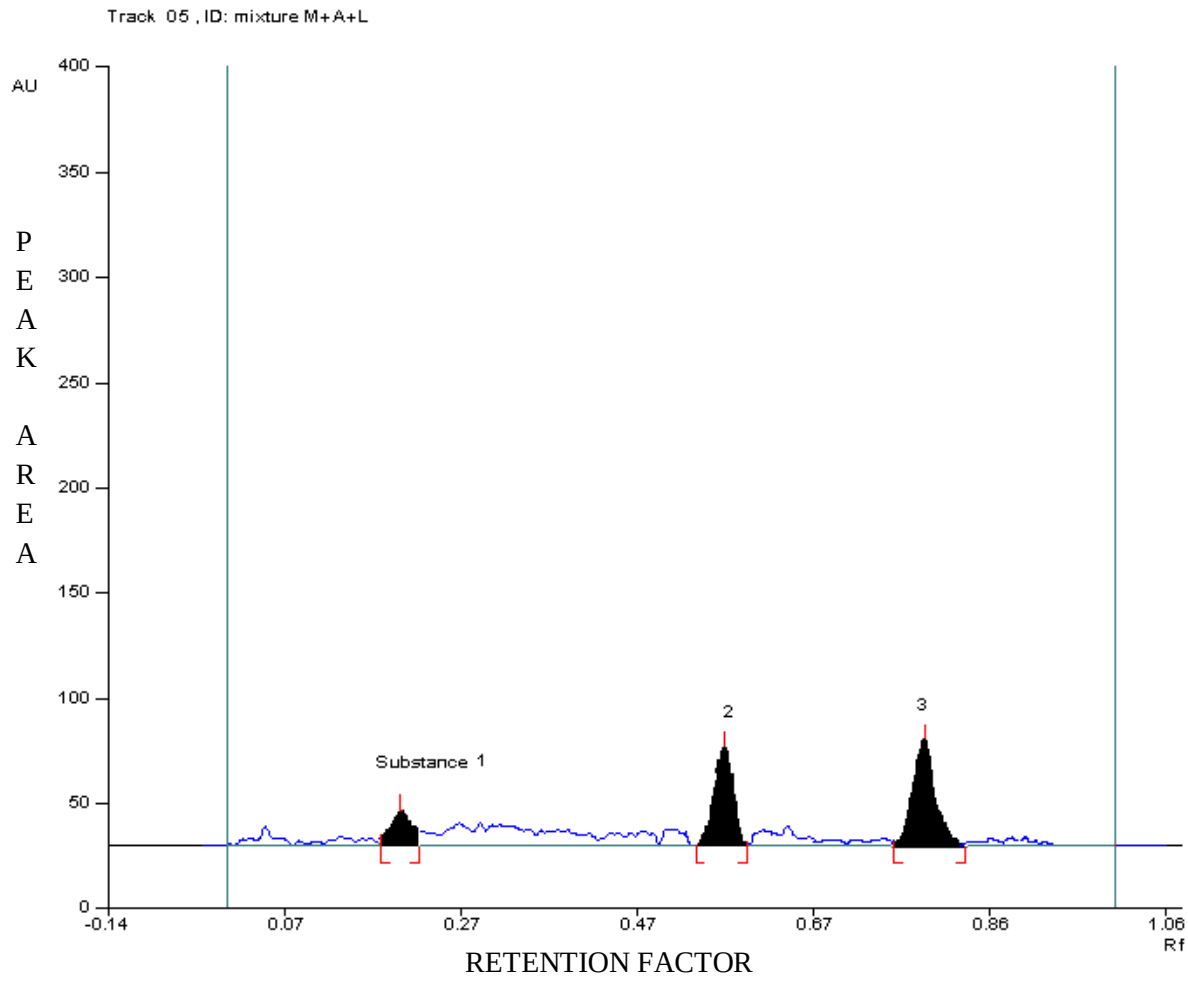
RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	13.4	25.77	0.22	2.9	256.6	21.34	LEVO
2.	0.56	2.6	0.59	32.1	44.01	0.61	1.4	771.7	36.2	MON
3.	0.76	1.4	0.78	46.9	37.36	0.79	0.3	1297.9	50.71	AMB

FIGURE – 36

ANALYSIS OF FORMULATION – RENEA

REPEATABILITY - 4

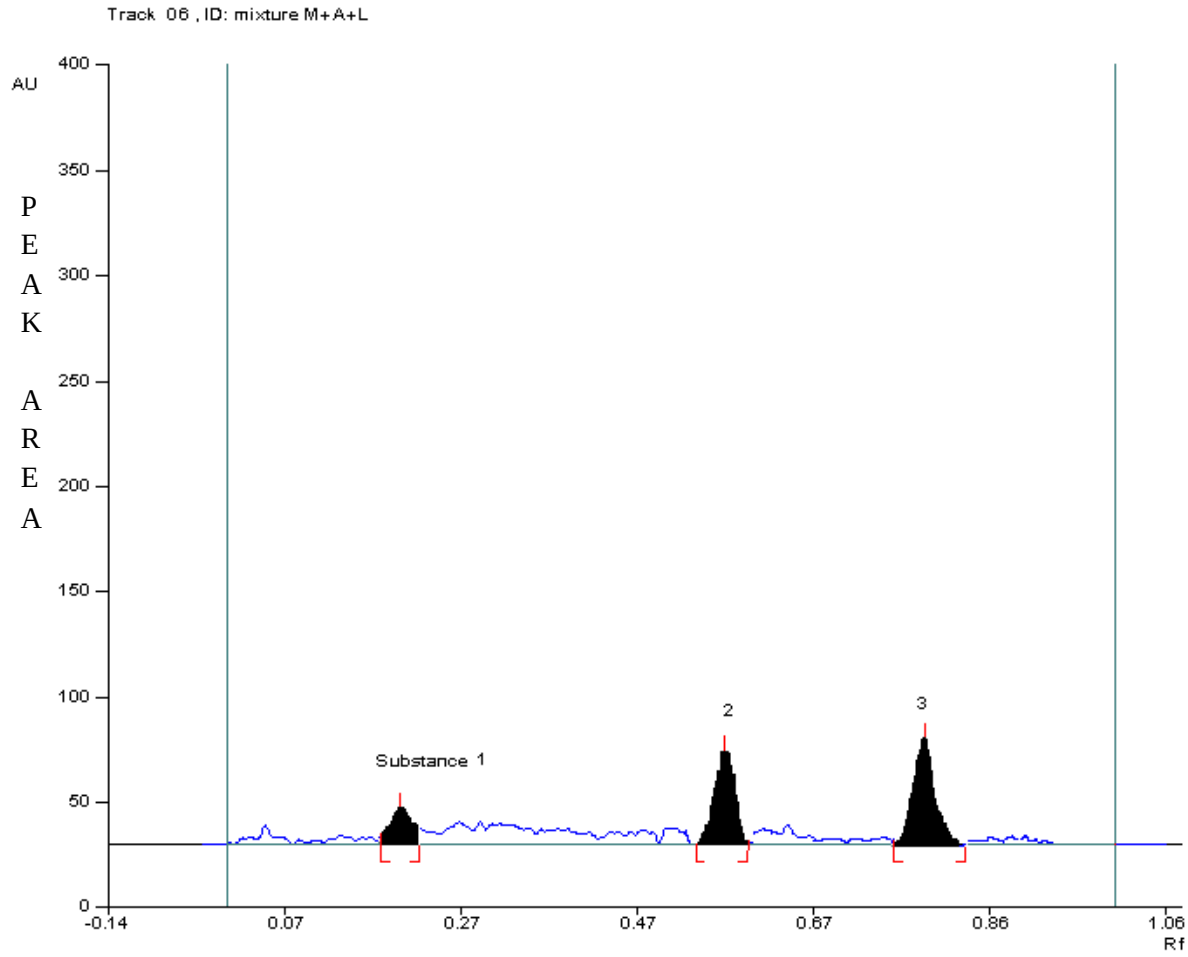


Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	15.1	25.77	0.22	2.2	260.6	19.32	LEVO
2.	0.56	2.6	0.59	32.1	44.01	0.61	0.8	766.7	35.21	MON
3.	0.76	1.4	0.78	47.9	37.36	0.79	1.7	1292.9	51.21	AMB

FIGURE – 37

ANALYSIS OF FORMULATION – RENEA

REPEATABILITY – 5



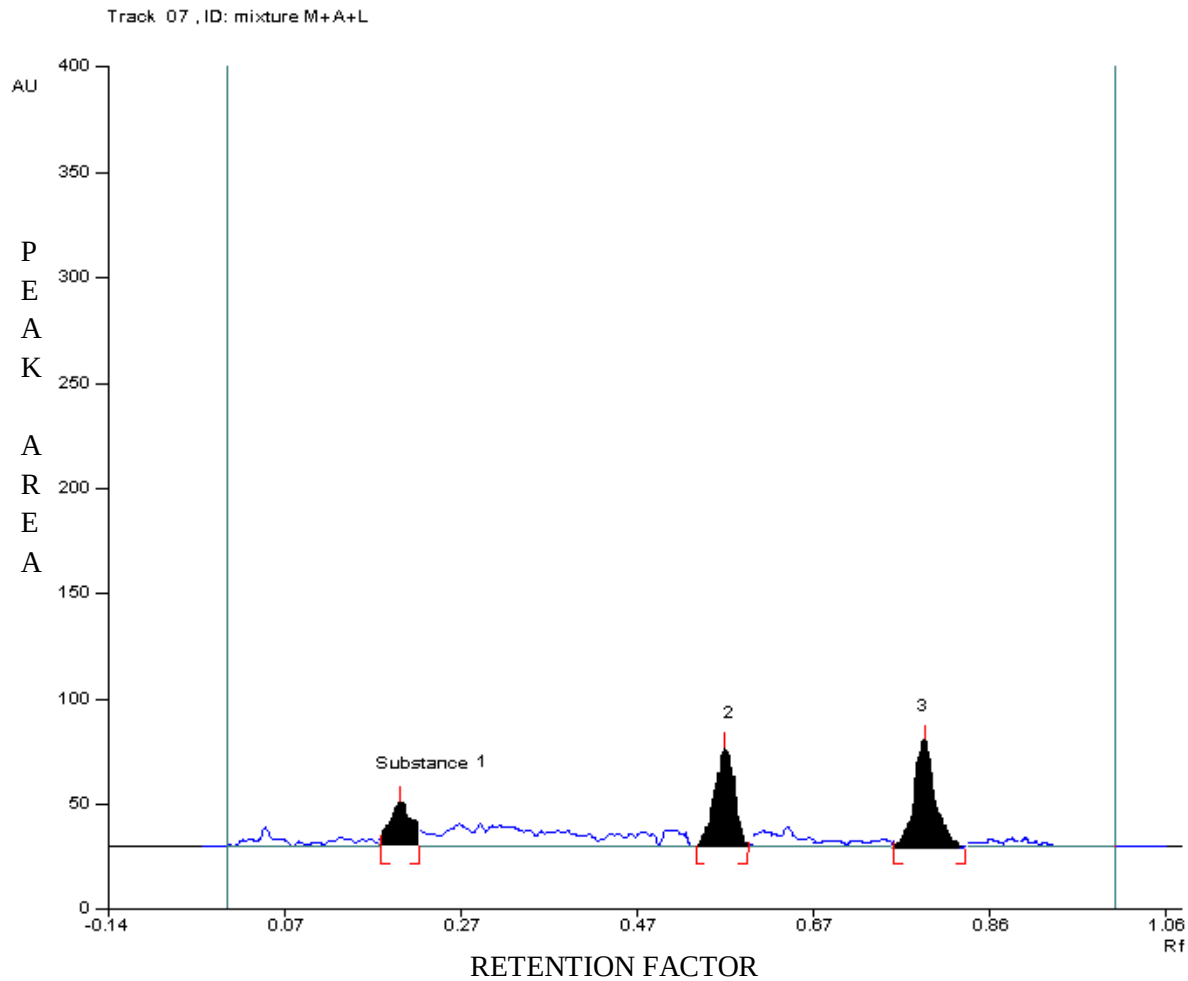
RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.20	15.6	25.77	0.22	0.9	261.2	20.65	LEVO
2.	0.56	2.6	0.59	32.1	44.01	0.61	1.1	769.9	35.21	MON
3.	0.76	1.4	0.78	49.9	37.36	0.79	1.3	1287.9	51.11	AMB

FIGURE – 38

ANALYSIS OF FORMULATION – RENEA

REPEATABILITY - 6

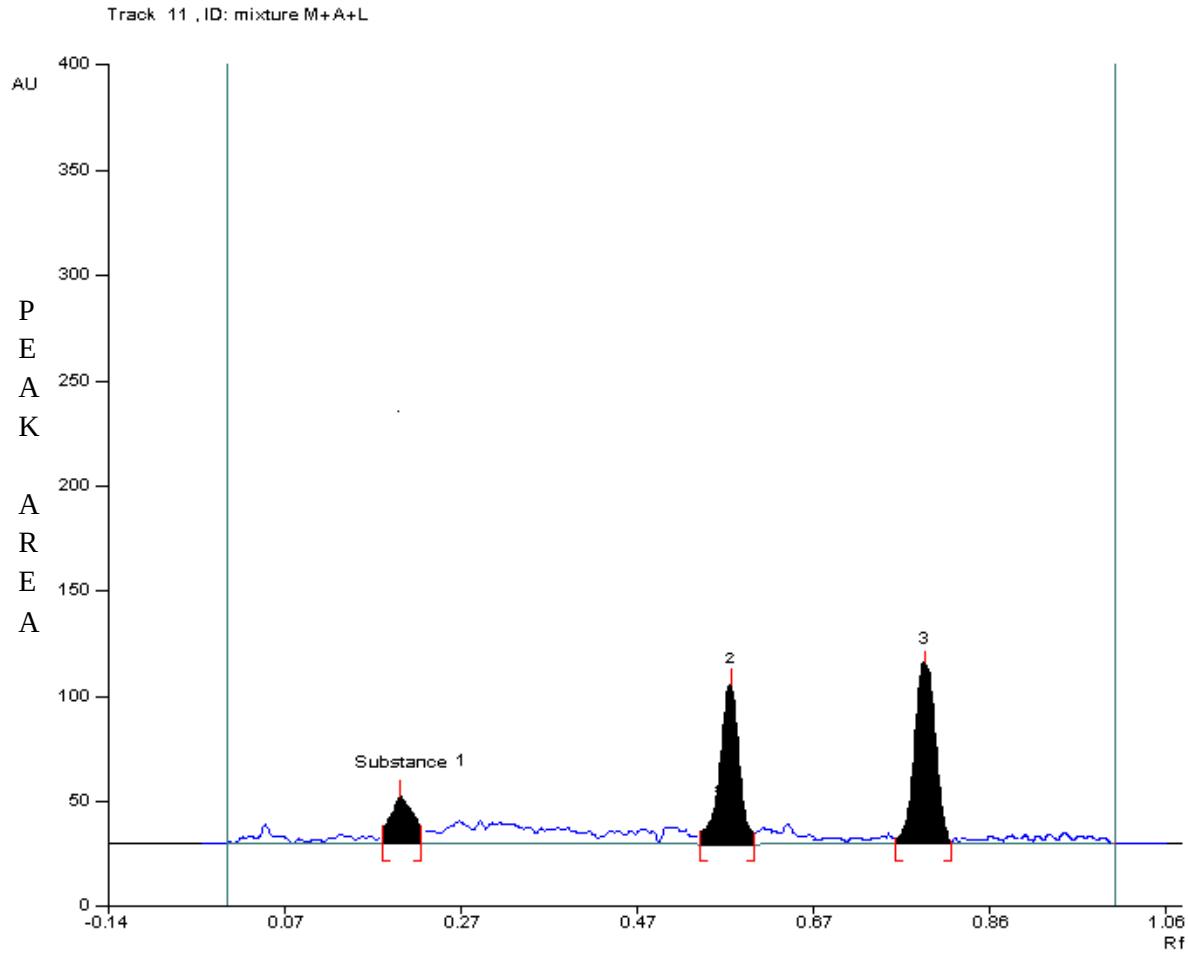


Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	13.9	25.77	0.22	0.2	262.3	20.31	LEVO
2.	0.56	2.6	0.59	32.1	44.01	0.61	1.2	769.1	35.31	MON
3.	0.76	1.4	0.78	45.6	37.36	0.79	0.76	1279.9	52.1	AMB

FIGURE – 39

RECOVERY ANALYSIS OF FORMULATION- RENEA

RECOVERY - 1



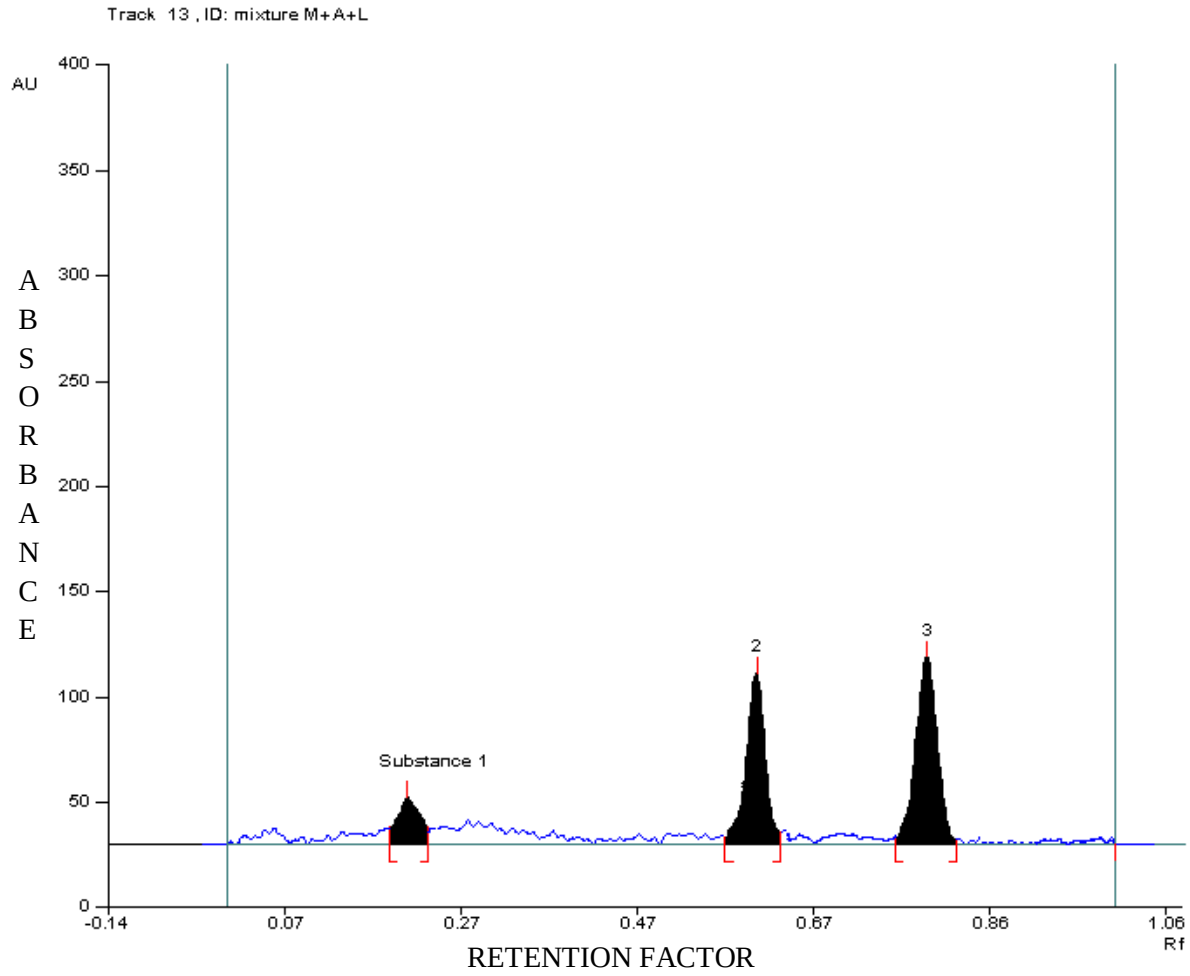
RETENTION FACTOR

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.20	17.6	16.83	0.24	4.2	487.54	15.31	LEVO
2.	0.56	2.6	0.59	35.21	35.21	0.63	1.2	1317.4	35.54	MON
3.	0.76	1.4	0.78	71.8	32.12	0.83	0.56	2292.23	50.32	AMB

FIGURE – 40

RECOVERY ANALYSIS OF FORMULATION- RENEA

RECOVERY - 2

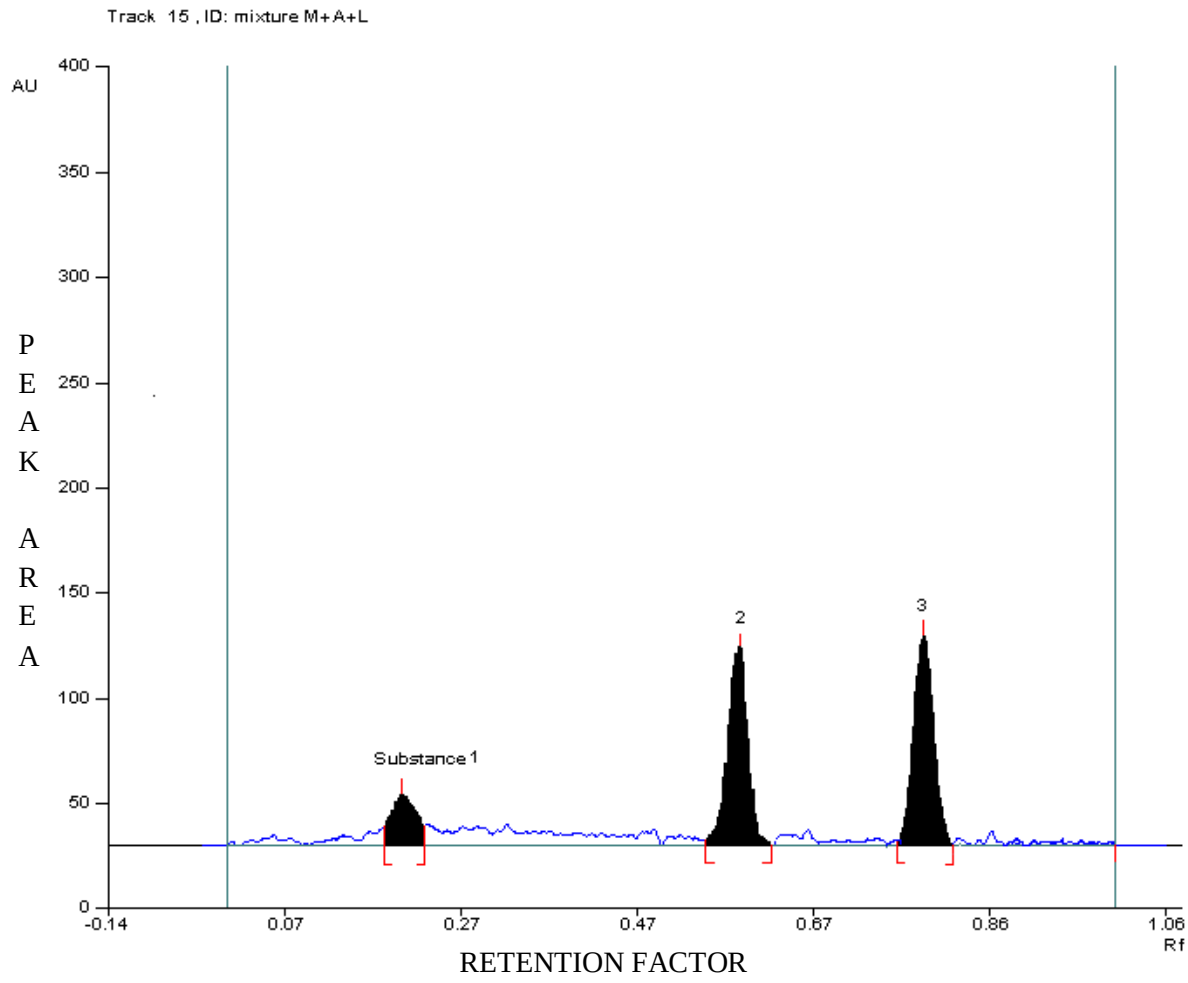


Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.21	18.6	25.77	0.22	1.2	509.89	14.21	LEVO
2.	0.56	2.6	0.59	81.4	44.01	0.61	2.6	1446.1	36.21	MON
3.	0.76	1.4	0.78	83.5	37.36	0.81	2.1	2543.54	50.21	AMB

FIGURE – 41

RECOVERY ANALYSIS OF FORMULATION- RENEA

RECOVERY - 3



Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Substance
1.	0.18	3.3	0.20	21.2	25.77	0.22	1.6	537.98	15.2	LEVO
2.	0.56	2.6	0.59	83.2	44.01	0.61	0.6	1586.2	34.21	MON
3.	0.76	1.4	0.78	96.3	37.36	0.79	1.2	2798.98	48.91	AMB

TABLES

TABLE-1

SOLUBILITY PROFILE OF MONTELUKAST SODIUM

IN POLAR AND NON-POLAR SOLVENTS

S.No.	SOLVENTS	EXTENT OF SOLUBILITY	CATEGORY
1	Distilled water	10 mg in 100µl	Freely soluble
2	0.1M Hydrochloric acid	10 mg in more than 10 ml	Insoluble
3	0.1M Sodium Hydroxide	10 mg in more than 10 ml	Insoluble
4	Methanol	10 mg in 20µl	Freely soluble
5	Ethanol	10 mg in 40µl	Freely soluble
6	Chloroform	10 mg in 30µl	Freely soluble
7	DMF	10 mg in 60µl	Freely soluble
8	Acetone	10 mg in 30µl	Freely soluble
9	Toluene	10 mg in 50µl	Freely soluble
10	n – Butanol	10 mg in 1.1 ml	Slightly soluble
11	Acetonitrile	10 mg in 40µl	Freely soluble
12	n – Hexane	10 mg in more than 10 ml	Insoluble
13	Isopropyl alcohol	10 mg in 1.2 ml	Slightly soluble
14	Ethyl acetate	10 mg in 60µl	Freely Soluble
15	Diethyl ether	10 mg in more than 10 ml	Insoluble
16	Pthalate buffer (pH 3.0)	10 mg in more than 10 ml	Insoluble
17	Pthalate buffer (pH 5.0)	10 mg in more than 10 ml	Insoluble
18	Phosphate buffer (pH 7.0)	10 mg in more than10 ml	Insoluble
19	Borate buffer (pH 9.0)	10 mg in1.9 ml	Slightly soluble
20	Benzene	10 mg in1.9 ml	Slightly Soluble
21	Dichloromethane	10 mg in 20µl	Freely soluble

TABLE-2

SOLUBILITY PROFILE OF LEVOCETIRIZINE DIHYDROCHLORIDE

IN POLAR AND NON-POLAR SOLVENTS

S.No.	SOLVENTS	EXTENT OF SOLUBILITY	CATEGORY
1	Distilled water	10 mg in 20µl	Freely soluble
2	0.1M Hydrochloric acid	10 mg in 50 µl	Freely soluble
3	0.1M Sodium Hydroxide	10 mg in more than 1 ml	Sparingly soluble
4	Methanol	10 mg in 20µl	Freely Soluble
5	Ethanol	10 mg in 60µl	Freely soluble
6	Chloroform	10 mg in more than10 ml	Insoluble
7	DMF	10 mg in 60µl	Freely soluble
8	Acetone	10 mg in more than 10 ml	Insoluble
9	Toluene	10 mg in more than 10 ml	Insoluble
10	n – Butanol	10 mg in 5 ml	Slightly Soluble
11	Acetonitrile	10 mg in more than 10 ml	Insoluble
12	Benzene	10 mg in more than 10 ml	Insoluble
13	Isopropyl alcohol	10 mg in more than 10 ml	In soluble
14	Dichloro methane	10 mg in more than 10 ml	In soluble
15	10% Glacial acetic acid	10 mg in 1.3 ml	Slightly soluble
16	Phthalate buffer (pH 3.0)	10 mg in ml 20 µl	Freely soluble
17	Phthalate buffer (pH 5.0)	10 mg in 40 µl	Freely soluble
18	Phosphate buffer (pH 7.0)	10 mg in 10 µl	Freely soluble
19	Borate buffer (pH 9.0)	10 mg in 60 µl	Freely soluble
20	Benzene	10 mg in more than 10 ml	Insoluble
21	Diethyl ether	10 mg of solute in 40 µl	Freely soluble

TABLE – 3

SOLUBILITY PROFILE OF AMBROXOL HYDROCHLORIDE

IN POLAR AND NONPOLAR SOLVENTS

S.No.	SOLVENTS	EXTENT OF SOLUBILITY	CATEGORY
1	Distilled water	10 mg in 600µl	Sparingly soluble
2	0.1M Hydrochloric acid	10 mg in 1 ml	Sparingly soluble
3	0.1M Sodium Hydroxide	10 mg in more than 10 ml	In soluble
4	Methanol	10 mg in 80µl	Freely soluble
5	Ethanol	10 mg in 500µl	Sparingly soluble
6	Chloroform	10 mg in more than 10 ml	In soluble
7	DMF	10 mg in 60µl	Freely soluble
8	Acetone	10 mg in more than 10 ml	Insoluble
9	Toluene	10 mg in more than 10 ml	Insoluble
10	n – Butanol	10 mg in 8 ml	Slightly Soluble
11	Acetonitrile	10 mg in more than 10 ml	Insoluble
12	n-Hexane	10 mg in more than 10 ml	Insoluble
13	Isopropyl alcohol	10 mg in 5 ml	Slightly soluble
14	Ethyl acetate	10 mg in more than 10 ml	In soluble
15	10% Glacial acetic acid	10 mg in 1 ml	Sparingly soluble
16	Phthalate buffer (pH 3.0)	10 mg in 1 ml	Sparingly soluble
17	Phthalate buffer (pH 5.0)	10 mg in 5 ml	Slightly soluble
18	Phosphate buffer (pH 7.0)	10 mg in 7 ml	Slightly soluble
19	Borate buffer (pH 9.0)	10 mg in 7 ml	Slightly soluble
20	Benzene	10 mg in more than 10 ml	Insoluble
21	Dichloro methane	10 mg in more than 10 ml	In soluble

TABLE - 4

OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM AT 345 nm BY ABSORBANCE CORRECTION METHOD

PARAMETERS	**MONTELUKAST SODIUM at 345 nm***
Beer's law limit (µg/ ml)	2 - 12
Molar Absorptivity ($L\ mol^{-1}\ cm^{-1)}$	28636.06094
Sandell's Sensitivity (µg/ cm^2/0.001 A.U.)	0.021560459
Correlation Coefficient (r)	0.9994
Regression Equation (y=mx+c)	Y=0.0467x+0.0031847
Slope (m)	0.0467671
Intercept (c)	0.00318476
LOD (µg/ ml)	0.6755608
LOQ (µg/ ml)	2.047154
Standard Error	0.00092495

* Mean of six observations

TABLE - 5

OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM AND AMBROXOL HYDROCHLORIDE AT 307 nm BY ABSORBANCE CORRECTION METHOD

PARAMETERS	MONTELUKAST SODIUM at 307 nm*	AMBROXOL HYDROCHLORIDE at 307 nm*
Beer's law limit (µg/ ml)	2 - 12	10 - 70
Molar Absorptivity (L mol^{-1} $cm^{-1)}$	13685.1577	2999.211464
Sandell's Sensitivity (µg/ cm^2/0.001 A.U.)	0.026588916	0.140949722
Correlation Coefficient (r)	0.9990	0.9994
Regression Equation (y=mx+c)	Y=0.0379x-0.000357	Y = 0.0071039+0.0013
Slope (m)	0.037961607	0.007103988
Intercept (c)	-0.00357616	0.0013
LOD (µg/ ml)	0.4205930	1.61590329
LOQ (µg/ ml)	1.261494	4.896676
Standard Error	0.00770206	0.00041593

* Mean of six observations

TABLE - 6

OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE AT 230 nm BY ABSORBANCE CORRECTION METHOD

PARAMETERS	MONTELUKAST SODIUM at 230 nm*	LEVOCETIRIZINE DIHYDROCHLORIDE at 230 nm*	AMBROXOL HYDROCHLORIDE at 230 nm*
Beer's law limit (µg/ ml)	2 – 12	1 – 12	10 - 70
Molar Absorptivity ($L\ mol^{-1}\ cm^{-1)}$	30329.57404	11876.57714	8796.3211
Sandell's Sensitivity (µg/ cm^2/0.001 A.U.)	0.0184409	0.033291836	0.0678888
Correlation Coefficient (r)	0.9991	0.9994	0.9993
Regression Equation (y=mx+c)	Y=0.0545x-0.0025	Y = 0.0300x+0.0049126	Y = 0.0147+0.00535
Slope (m)	0.05454	0.0030048413	0.001473956
Intercept (c)	-0.00252916	0.00491267	0.00535015
LOD (µg/ ml)	0.413010	0.0528027375	1.1978301
LOQ (µg/ ml)	1.25254	0.16000829	3.629788
Standard Error	0.010383	0.000482027	0.0141386

* Mean of six observations

TABLE – 7

QUANTIFICATION OF FORMULATION (RENEA)

BY ABSORBANCE CORRECTION METHOD

Drug	Sample No.	Labelled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%) ± SD	% RSD	SE	CI@
MON	1	10	10.11	101.1	100.45 ± 0.6253	0.6224	0.0173	99.79 to 101.10
	2	10	10.11	101.1				
	3	10	10.05	100.5				
	4	10	10.05	100.5				
	5	10	10.00	100.00				
	6	10	9.95	99.5				
LEVO	1	5	5.09	101.83	100.38 ± 1.74261	1.7359	0.0484	98.55 to 102.20
	2	5	4.94	98.8				
	3	5	5.02	100.05				
	4	5	4.90	98.06				
	5	5	5.05	101				
	6	5	5.12	102.55				
AMB	1	75	73.75	98.33	100.32 ± 1.1773	1.1735	0.0327	99.08 to 101.55
	2	75	75.2	100.26				
	3	75	75.42	100.50				
	4	75	75.81	101.08				
	5	75	76.36	101.81				
	6	75	74.96	99.95				

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride

* Mean of six observations

CI@ = confidence interval (95 %)

TABLE – 8

INTRADAY AND INTERDAY ANALYSIS OF FORMULATION (RENEA) BY ABSORBANCE CORRECTION METHOD

Drug	Sample No.	Labelled amount (mg/tab)	Percentage obtained*		± SD		% RSD	
			Intraday	Interday	Intra day	Inter day	Intra day	Inter day
MON	1	10	100.1	100.1	0.2309	0.2645	0.2310	0.2651
	2	10	99.7	99.6				
	3	10	100.1	99.7				
Mean			99.96	99.8				
Confidence interval (95 %)			99.71-100.20	99.52-100.07				
LEVO	1	5	102.3	102.4	0.6245	0.3464	0.6116	0.3376
	2	5	102.6	103				
	3	5	101.4	102.4				
Mean			102.1	102.6				
Confidence interval (95 %)			101.4-102.75	102.23-103.61				
AMB	1	75	102.5	101.52	0.8888	0.0305	0.8731	0.0300
	2	75	100.8	101.56				
	3	75	102.1	101.58				
Mean			101.8	101.55				
Confidence interval (95 %)			100.86-102.73	101.51-101.58				

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations

TABLE – 9

RUGGEDNESS STUDY OF FORMULATION (RENEA) BY ABSORBANCE CORRECTION METHOD (DIFFERENT ANALYST)

S. No	Drug	Condition	Mean %*	± SD	% RSD	SE	CI[@]
1	Montelukast Sodium	Analyst 1	100.45	0.6253	0.6224	0.0173	99.79 to 101.10
2		Analyst 2	99.6	0.4648	0.4666	0.0729	99.11 to 100.08
3	Levocetirizine Dihydrochloride	Analyst 1	100.38	1.7426	1.7359	0.0484	98.55 to 102.20
4		Analyst 2	101.74	1.9288	1.8957	0.0535	99.71 to 103.76
5	Ambroxol Hydrochloride	Analyst 1	100.32	1.1773	1.1735	0.0327	99.08 to 101.55
6		Analyst 2	101.68	0.4739	0.4660	0.0131	101.18 to 102.17

*Mean of six observations

CI[@] = confidence interval (95 %)

TABLE – 10

RUGGEDNESS STUDY OF FORMULATION (RENEA) BY ABSORBANCE CORRECTION METHOD (DIFFERENT INSTRUMENT)

S. No	Drug	Condition	Mean %*	± SD	% RSD	SE	CI@
1	Montelukast Sodium	Instrument 1	99.32	0.5980	0.6026	0.0166	98.69 to 99.94
2		Instrument 2	99.98	1.0186	1.0188	0.0282	98.91 to 101.05
3	Levocetirizine Dihydrochloride	Instrument 1	101.50	0.8431	0.8306	0.0234	100.61 to 102.38
4		Instrument 2	101.89	0.7640	0.7499	0.0212	101.08 to 102.69
5	Ambroxol Hydrochloride	Instrument 1	100.01	1.2708	1.2706	0.0353	98.07 to 101.34
6		Instrument 2	99.65	1.1617	1.1656	0.0332	98.43 to 100.87

*Mean of six observations
CI@ = confidence interval (95 %)

TABLE – 11

RECOVERY ANALYSIS OF FORMULATION – RENEA BY ABSORBANCE CORRECTION METHOD (80 % Recovery)

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount recovered (µg/ml)	% Recovered*	SD	% RSD
MON	1	6.94	2.91	2.92	100.34	0.8111	0.8121
	2	6.87	2.84	2.85	100.35		
	3	6.83	2.84	2.81	98.94		
				Mean	99.87		
				CI@	99.01 – 100.72		
LEVO	1	3.53	1.51	1.52	100.66	1.4764	1.4907
	2	3.52	1.53	1.51	98.69		
	3	3.33	1.35	1.32	97.77		
				Mean	99.04		
				CI@	97.49 – 100.58		
AMB	1	53.85	24.05	23.78	98.87	0.4986	0.5047
	2	53.05	23.05	23.05	99.26		
	3	54.43	24.51	24.51	98.27		
				Mean	98.8		
				CI@	98.27 – 99.32		

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations
CI@ = confidence interval (95 %)

TABLE – 12

RECOVERY ANALYSIS OF FORMULATION – RENEA

BY ABSORPTION CORRRECTION METHOD (100 % Recovery)

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount recovered (µg/ml)	% Recovered*	SD	% RSD
MON	1	8.49	4.52	4.47	98.89	0.3404	0.3445
	2	8.53	4.55	4.51	99.12		
	3	8.49	4.54	4.47	98.45		
				Mean	98.82		
				CI@	98.46 – 99.17		
LEVO	1	4.92	2.88	2.91	101.04	1.3900	1.3857
	2	4.80	2.80	2.79	99.64		
	3	4.97	2.89	2.96	102.42		
				Mean	101.03		
				CI@	99.57 – 102.48		
AMB	1	59.80	29.08	29.83	102.57	0.7261	0.7137
	2	60.06	29.72	30.09	101.24		
	3	60.34	29.95	30.37	101.40		
				Mean	101.73		
				CI@	100.96 – 102.49		

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations
CI@ = confidence interval (95 %)

TABLE – 13

RECOVERY ANALYSIS OF FORMULATION – RENEA

BY ABSORBANCE CORRECTION METHOD (120 % Recovery)

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount recovered (µg/ml)	% Recovered*	SD	% RSD
MON	1	9.97	6.02	5.95	98.83	0.5875	0.5912
	2	9.95	5.97	5.93	99.32		
	3	9.95	5.93	5.93	100		
				Mean	99.38		
				CI[@]	98.76 – 99.17		
LEVO	1	6.59	4.49	4.58	102.00	1.4733	1.4625
	2	6.56	4.59	4.55	99.12		
	3	6.59	4.53	4.58	101.10		
				Mean	100.74		
				CI[@]	99.19 – 102,28		
AMB	1	66.08	36.18	36.11	99.80	0.4452	0.4441
	2	66.17	36.20	36.20	100.22		
	3	66.24	36.27	36.27	100.69		
				Mean	100.23		
				CI[@]	99.76 – 100.69		

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations
CI[@] = confidence interval (95 %)

TABLE- 14

OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM AT 365.5 nm BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY

PARAMETERS	**MONTELUKAST SODIUM at 365.5 nm***
Beer's law limit (µg/ml)	2 - 12
Molar Absorptivity ($L\ mol^{-1}\ cm^{-1)}$	1589.63088
Sandell's Sensitivity (µg/ cm^2/0.001 A.U.)	0.381473795
Correlation Coefficient (r)	0.9992
Regression Equation (y=mx+c)	Y=0.0026x+(-0.00028)
Slope (m)	0.00264273
Intercept (c)	-0.0002894
LOD (µg/ml)	0.69616629
LOQ (µg/ml)	2.0883827
Standard Error	0.00009362

*Mean of six observations

TABLE- 15

OPTICAL CHARACTERISTICS OF AMBROXOL HYDROCHLORIDE AT 256.5 nm BY FIRST ORDER DERIVATIVE SPECTROSCOPY

PARAMETERS	**AMBROXOL HYDROCHLORIDE at 256.5 nm***
Beer's law limit (µg/ml)	10 - 70
Molar Absorptivity ($L\ mol^{-1}\ cm^{-1)}$	726.528916
Sandell's Sensitivity ($\mu g/\ cm^2$/0.001 A.U.)	0.5697066
Correlation Coefficient (r)	0.9989
Regression Equation (y= mx+c)	Y = 0.001761+0.00009027
Slope (m)	0.00176138
Intercept (c)	0.000090277
LOD (µg/ml)	1.728272
LOQ (µg/ml)	5.237189
Standard Error	0.0001965

*Mean of six observations

TABLE- 16

OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM AND LEVOCETIRIZINE HYDROCHLORIDE AT 248 nm BY FIRST ORDER DERIVATIVE SPECTROSCOPY

PARAMETERS	MONTELUKAST SODIUM at 248 nm*	LEVOCETIRIZINE DIHYDROCHLORIDE at 248 nm*
Beer's law limit (µg/ml)	2 - 12	2 - 12
Molar Absorptivity ($L\ mol^{-1}\ cm^{-1)}$)	372.832369	259.3062
Sandell's Sensitivity (µg/ cm^2/0.001 A.U.)	1.503564	1.50551854
Correlation Coefficient (r)	0.9993	0.9993
Regression Equation (y=mx+c)	Y=0.0006x+(-0.000016)	Y = 0.00066x+0.000013690
Slope (m)	0.000669	0.0006681
Intercept (c)	-0.00001666	0.000013690
LOD (µg/ml)	0.4568765	0.4483850
LOQ (µg/ml)	1.384474	1.3587425
Standard Error	0.000116	0.000012426

*Mean of six observations

TABLE – 17

QUANTIFICATION OF FORMULATION (RENEA)
BY FIRST ORDER DERIVATIVE SPECTROSCOPY

Drug	Sample No.	Labelled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%) ± SD	% RSD	SE	CI@
MON	1	10	10.06	100.6	99.2 ± 0.8854	0.8925	0.0245	98.27 to 100.12
	2	10	9.99	99.9				
	3	10	9.85	98.5				
	4	10	9.92	99.2				
	5	10	9.85	98.5				
	6	10	9.85	98.5				
LEVO	1	5	5.04	101.6	101.21 ± 0.8900	0.8794	0.0247	100.27 to 102.14
	2	5	5.14	102.8				
	3	5	5.03	100.57				
	4	5	5.06	101.2				
	5	5	5.02	100.55				
	6	5	5.02	100.55				
AMB	1	75	75.47	100.62	99.82 ± 1.0461	1.0479	0.0290	98.72 to 100.91
	2	75	75.9	100.93				
	3	75	74.97	99.99				
	4	75	75.13	100.17				
	5	75	74.34	99.12				
	6	75	73.64	98.1				

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of six observations
CI@ = confidence interval (95 %)

TABLE – 18

INTRADAY AND INTERDAY ANALYSIS OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROSCOPY

Drug	Sample No.	Labelled amount (mg/tab)	Percentage obtained*		± SD		% RSD	
			Intraday (mg)	Interday (mg)	Intra day	Inter day	Intra day	Inter day
MON	1	10	98.8	98.9	0.5253	0.0115	0.5296	0.0116
	2	10	99.8	98.8				
	3	10	98.8	98.9				
Mean			99.19	98.89				
Confidence interval (95 %)			98.63-99.74	99.67-99.90				
LEVO	1	5	100.89	102.8	0.7678	0.1154	0.7579	0.1123
	2	5	102.2	102.6				
	3	5	100.89	102.8				
Mean			101.31	102.73				
Confidence interval (95 %)			100.50-102.11	102.60-102.85				
AMB	1	75	100.48	99.79	1.2951	1.2419	1.2816	1.2361
	2	75	100.15	101.9				
	3	75	102.54	99.71				
Mean			101.05	100.46				
Confidence interval (95 %)			99.69-102.409	99.15-101.76				

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations

TABLE – 19

RUGGEDNESS STUDY OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROSCOPY (DIFFERENT ANALYST)

S. No	Drug	Condition	Mean %*	± SD	% RSD	SE	CI@
1	Montelukast Sodium	Analyst 1	99.2	0.8854	0.8925	0.0245	98.27 to 100.12
2		Analyst 2	99.16	0.7076	0.7136	0.0196	98.41 to 99.90
3	Levocetirizine Dihydrochloride	Analyst 1	101.21	0.8900	0.8794	0.0247	100.27 to 102.14
4		Analyst 2	101.14	1.8824	1.8801	0.0522	99.16 to 103.11
5	Ambroxol Hydrochloride	Analyst 1	99.82	1.0461	1.0479	0.0290	98.72 to 100.19
6		Analyst 2	99.45	0.7324	0.7363	0.0203	98.68 to 100.21

*Mean of six observations

CI@ = confidence interval (95 %)

TABLE – 20

RUGGEDNESS STUDY OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROSCOPY (DIFFERENT INSTRUMENT)

S. No	Drug	Condition	Mean %*	± SD	% RSD	SE	CI[@]
1	Montelukast Sodium	Instrument 1	99.35	0.7007	0.7053	0.0194	98.61 to 100.08
2		Instrument 2	100.15	1.1023	1.1018	0.0306	98.99 to 101.30
3	Levocetirizine Dihydrochloride	Instrument 1	100.90	1.7913	1.7752	0.0497	99.02 to 102.77
4		Instrument 2	101.19	0.8402	0.8363	0.0235	100.30 to 102.07
5	Ambroxol Hydrochloride	Instrument 1	100.40	0.8096	0.8063	0.0224	99.55 to 101.24
6		Instrument 2	100.85	1.1940	1.1839	0.0331	99.59 to 102.10

*Mean of six observations

CI[@] = confidence interval (95 %)

TABLE – 21

RECOVERY ANALYSIS OF FORMULATION (RENEA)

BY FIRST ORDER DERIVATIVE SPECTROSCOPY (80 % Recovery)

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount recovered (µg/ml)	% Recovered*	SD	% RSD
MON	1	6.99	2.99	2.99	100	1.2572	1.2756
	2	6.94	3.01	2.94	98		
	3	6.95	3.02	2.95	97.68		
				Mean	98.56		
				CI@	97.24 – 99.87		
LEVO	1	3.48	1.50	1.48	98.66	0.1154	0.1171
	2	3.28	1.30	1.28	98.46		
	3	3.28	1.30	1.28	98.46		
				Mean	98.52		
				CI@	98.39 – 98.64		
AMB	1	51.62	21.72	21.62	99.53	0.3119	0.3139
	2	49.98	20.07	19.98	99.55		
	3	49.96	20.16	19.96	99		
				Mean	99.36		
				CI@	99.03 – 99.68		

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations
CI@ = confidence interval (95 %)

TABLE – 22

RECOVERY ANALYSIS OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROSCOPY (100 % Recovery)

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount recovered (µg/ml)	% Recovered*	SD	% RSD
MON	1	8.42	4.48	4.44	99.10	0.4450	0.4490
	2	8.46	4.50	4.48	99.55		
	3	8.42	4.50	4.44	98.66		
				Mean	99.10		
				CI[@]	98.63 – 99.56		
LEVO	1	4.92	2.94	2.90	98.63	2.0612	2.0524
	2	5.06	2.99	3.00	103.4		
	3	4.93	2.94	2.91	98.97		
				Mean	100.33		
				CI[@]	98.16 – 102.49		
AMB	1	57.99	28.42	27.94	98.81	0.9930	1.0163
	2	58.36	29.06	28.31	97.91		
	3	58.65	29.56	28.6	96.89		
				Mean	99.76		
				CI[@]	98.71 – 100.80		

MON=Montelukast Sodium, LEVO=Levocetirizine Dihydrochloride,
AMB=Ambroxol Hydrochloride
*Mean of three observations
CI[@] = confidence interval (95 %)

TABLE – 23

RECOVERY ANALYSIS OF FORMULATION (RENEA) BY FIRST ORDER DERIVATIVE SPECTROSCOPY (120 % Recovery)

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount recovered (µg/ml)	% Recovered*	SD	% RSD
MON	1	9.86	5.94	5.88	98.98	0.2771	0.2808
	2	9.91	6.02	5.93	98.50		
	3	9.90	6.01	5.92	98.50		
				Mean	98.66		
				CI[@]	98.36 – 98.95		
LEVO	1	6.37	4.41	4.35	98.63	1.3975	1.4041
	2	6.43	4.37	4.42	99.37		
	3	6.21	4.42	4.19	98.71		
				Mean	99.53		
				CI@	98.06 – 100.99		
AMB	1	65.29	36.26	35.24	97.18	1.1234	1.1414
	2	64.86	35.03	34.81	99.37		
	3	64.51	34.91	34.46	98.71		
				Mean	98.42		
				CI[@]	97.24 – 99.59		

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations
CI[@] = confidence interval (95 %)

TABLE- 24

OPTICAL CHARACTERISTICS OF MONTELUKAST SODIUM, LEVOCETIRIZINE DIHYDROCHLORIDE AND AMBROXOL HYDROCHLORIDE BY HPTLC

PARAMETERS	MONTELUKAST SODIUM	LEVOCETIRIZINE DIHYDROCHLORIDE	AMBROXOL HYDROCHLORIDE
Detection wavelength	224 nm	224 nm	224 nm
Linearity range (ng/µl)	20 - 200	20 - 200	75 - 750
Correlation Coefficient (r)	0.9992	0.9990	0.9993
Regression Equation (y=mx+c)	Y=17.3154x+63.7566	Y = 12.6776x+(-6.4515)	Y = 4.1748x+11.5865
Slope (m)	17.315487	12.677624	4.174865
Intercept (c)	63.756667	-6.451515	11.586515
LOD (ng/µl)	1.0238	2.1260	6.7883
LOQ (ng/µl)	3.1025	6.4424	20.5708
Standard Error	3.8085	0.9013	1.8381

TABLE – 25

QUANTIFICATION OF FORMULATION (RENEA)
BY HPTLC

Drug	Sample No.	Labelled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%) ± SD	% RSD	SE	CI@
MON	1	10	10.018	100.18	99.97 ± 0.4881	0.4882	0.0134	99.45 to 100.48
	2	10	10.075	100.75				
	3	10	10.004	100.04				
	4	10	9.934	99.34				
	5	10	9.979	99.79				
	6	10	9.967	99.67				
LEVO	1	5	4.975	99.5	101.89 ± 1.6881	1.6567	0.0468	100.11 to 103.66
	2	5	5.013	100.26				
	3	5	5.09	101.8				
	4	5	5.15	103				
	5	5	5.16	103.2				
	6	5	5.18	103.6				
AMB	1	75	75.452	100.6	100.02 ± 0.5487	0.5486	0.0152	98.39 to 101.64
	2	75	75.042	100.05				
	3	75	75.394	100.52				
	4	75	75.101	100.13				
	5	75	74.808	99.74				
	6	75	74.339	99.11				

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of six observations
CI@ = confidence interval (95 %)

TABLE – 26

INTRADAY AND INTERDAY ANALYSIS OF FORMULATION (RENEA) BY HPTLC

Drug	Sample No.	Labelled amount (mg/tab)	Percentage obtained*		± S.D		% R.S.D.	
			Intraday (mg)	Inter day (mg)	Intra day	Inter day	Intra day	Inter day
MON	1	10	99.79	99.77	0.2330	0.5225	0.2379	0.5234
	2	10	99.34	100.38				
	3	10	99.67	99.34				
Mean			99.60	99.83				
Confidence interval			99.71-100.70	99.61-100.62				
LEVO	1	5	101.8	101.76	1.1718	0.3233	1.1657	0.3168
	2	5	99.5	102				
	3	5	100.26	102.4				
Mean			100.52	102.05				
Confidence interval			99.44-101.95	101.91-102.31				
AMB	1	75	100.05	100.05	0.2514	0.3265	0.2508	0.3269
	2	75	100.13	100.08				
	3	75	100.52	99.5				
Mean			100.23	99.87				
Confidence interval			100.06-101.03	99.13-100.12				

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations

TABLE – 27

RECOVERY ANALYSIS OF FORMULATION (RENEA)

BY HPTLC (80 % Recovery)

Drug	Sample No	Amount present (ng/µl)	Amount added (ng/µl)	Amount recovered (ng/µl)	% Recovered*	SD	% RSD
LEVO	1	36.9656	16	16.1319	100.73	0.6854	0.6872
	2	36.7216	16	15.8881	99.37		
	3	36.8162	16	15.9825	99.90		
				Mean	100.00		
				CI@	99.28 – 100.71		
MON	1	72.4001	32	31.5478	98.58	0.9086	0.9115
	2	71.9316	32	31.0858	97.14		
	3	71.8621	32	31.0098	96.90		
				Mean	97.54		
				CI@	96.58 – 98.49		
AMB	1	546.2793	240	239.6874	99.87	0.4650	0.4663
	2	547.4460	240	240.8541	100.35		
	3	545.2160	240	238.6241	99.42		
				Mean	99.88		
				CI@	99.39 – 100.36		

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations
CI@ = confidence interval (95 %)

TABLE – 28

RECOVERY ANALYSIS OF FORMULATION – RENEA

BY HPTLC (100 % Recovery)

Drug	Sample No	Amount present (ng/µl)	Amount added (ng/µl)	Amount recovered (ng/µl)	% Recovered*	SD	% RSD
LEVO	1	40.7285	20	19.8945	99.47	1.9744	2.0024
	2	40.8320	20	19.9989	99.99		
	3	40.1031	20	19.2694	96.34		
				Mean	98.60		
				CI[@]	96.52 – 100.67		
MON	1	79.8327	40	38.9804	97.45	1.3291	1.3417
	2	80.8527	40	40.0004	100		
	3	80.1021	40	39.2498	98.12		
				Mean	98.54		
				CI[@]	97.14 – 99.93		
AMB	1	606.4750	300	299.8831	99.96	0.9586	0.9657
	2	605.5963	300	299.0044	99.66		
	3	601.1230	300	299.5311	98.17		
				Mean	99.26		
				CI[@]	98.25 – 100.26		

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations
CI[@] = confidence interval (95 %)

TABLE – 29

RECOVERY ANALYSIS OF FORMULATION – RENEA

BY HPTLC (120 % Recovery)

Drug	Sample No	Amount present (ng/µl)	Amount added (ng/µl)	Amount recovered (ng/µl)	% Recovered*	SD	% RSD
LEVO	1	44.9442	24	24.1105	100.50	0.7633	0.7657
	2	44.7352	24	23.9015	99.55		
	3	44.6121	24	23.7784	98.99		
				Mean	99.68		
				CI[@]	98.87 – 100.47		
MON	1	87.9237	48	47.0714	98.06	0.7494	0.7595
	2	88.0970	48	47.2447	98.42		
	3	88.6136	48	47.7613	99.50		
				Mean	98.66		
				CI[@]	97.87 – 99.44		
AMB	1	667.6606	360	361.0741	100.29	0.3517	0.3613
	2	665.5049	360	358.9125	99.69		
	3	666.7160	360	360.1241	100.34		
				Mean	100.10		
				CI[@]	99.73 – 100.46		

MON = Montelukast Sodium, LEVO = Levocetirizine Dihydrochloride,
AMB = Ambroxol Hydrochloride
*Mean of three observations
CI[@] = confidence interval (95 %)

BIBLIOGRAPHY

7. BIBLIOGRAPHY

1. Aidong Wen, Taijun Hang, Suning Chen and Zhirui Wang. Simultaneous Determination of Amoxicillin and Ambroxol in Human Plasma by LC-MS/MS: Validation and Application to Pharmacokinetic study, *Journal of Pharmaceutical and Biomedical Analysis*, **2008**, 48 (3), 829 - 834.
2. Al Omari Mohmoud M., Zoubi Rufaida M., Hasan Enas I., Khader tariq Z. and Badwan Adnan A. Effect of Light and Heat on the Stability of Montelukast in Solution and in its Solid state, *Journal of Pharmaceutical and Biomedical Analysis*, **2007**, 45 (3), 465 - 471.
3. Al-Rawithi Sameer, Al-Gazian Sulaiman, Al-Ahmadi Wijdan, Alshowaier Ibrahim A., Yusuf Ahmed and Raines Dale A. Expedient Liquid Chromatographic Method with Fluorescence Detection for Montelukast Sodium in Micro - Samples of Plasma, *Journal of Chromatography B: Biomedical Sciences and Applications*, **2001**, 754 (2), 527 - 531.
4. Ambadas R. Rote and Vaishali S. Niphade. Determination of Montelukast Sodium and Levocetirizine Dihydrochloride in Combined Tablet Dosage form by HPTLC and First - derivative spectrophotometry, *Journal of Liquid Chromatography and Related technologies*, **2011,** 34 (3), 155 - 167.
5. Anjaneyulu Y., Chandra sekhar K. and Valli Manickam. A Text Book of Analytical Chemistry, Pharmamed Press, Hyderabad, **2006,** 1 – 4.
6. Anonymous. Clarke's Analysis of Drugs and Poisons, 3rd edition, The Pharmaceutical Press, A Division of the Royal Pharmaceutical Society of Great Britain, London, **2004**, 770, 1299.

7. Anonymous. Elico Instruction Manual Pharamaspec SL – 210 Series Operation Guide, Elico Limited, Hyderabad, India, **2009**, C – 2.
8. Anonymous. http://www.chemistry.msu.edu/faculty/reusch/virtTxtJml/Spectrpy/UV Vis/uvspec.html
9. Anonymous. http://www.drugs.com/mtm/montelukast.html
10. Anonymous. http://www.en.wikepedia.org/wiki/Monte.html
11. Anonymous. http://www.en.wikipedia.org/wiki/Ambroxol.html
12. Anonymous. http://www.en.wikipedia.org/wiki/Levocetirizine.html
13. Anonymous. Martindale, The Extra Pharmacopoeia, 30th edition, The Pharmaceutical Press, London, **1993,** 743.
14. Anonymous. Shimadzu Instruction Manual AX - 200 Digital Balance, Shimadzu Corporation, Kyoto, Japan, **2001,** 42.
15. Anonymous. Shimadzu Instruction Manual Pharamaspec UV – 1700 Series Operation Guide, Shimadzu Corporation, Kyoto, Japan, **2001**, 6.2.
16. Anonymous. The British Pharmacopoeia Volume I, International edition, Office of British Pharmacopoeia Commission, London, **2009,** 265.
17. Anonymous. The Indian Pharmacopoeia volume I and volume II, The Indian Pharmacopoeia Commission, Ghaziabad, **2007**, 142-143, 672 - 674,701.
18. Anonymous. The Merck Index, 14th edition, Merck Research Laboratories a Division of Merck & Co. Inc, White house Station, NJ, USA, **2006**, 66, 334.
19. Arayne M.S., Sultana N., Mirza A.Z. and Siddiqui F.A. Simultaneous Determination of Gliquidone, Fexofenadine, Buclizine and Levocetirizine in Dosage Formulation and Human Serum by RP-HPLC, *Journal of Chromatographic Sciences*, **2010**, 48 (5), 382 - 385.

20. Arindam Basu, Krishnendu Basak, Mithun Chakraborty and Inder Singh Rawat. Simultaneous RP-HPLC Estimation of Levocetirizine Hydrochloride and Montelukast Sodium in Tablet Dosage Form, *International Journal of Pharm Tech Research*, **2011**, 3 (1), 405 – 410.
21. Ashok Kumar S., Senthil Raja M. and Perumal P. RP - HPLC Method Development and Validation for Simultaneous Estimation of Montelukast Sodium and Levocetirizine Dihydrochloride, *International Journal of Pharmaceutical Research*, **2009**, 1 (4), 8 - 12.
22. Balasekhara Reddy Challa, Bahlul Z. Awen, Babu Rao Chandu, Mukkanti Khagga and Chandrasekhar Bannoth Kotthapalli. Method Development and Validation of Montelukast in Human Plasma by HPLC Coupled with ESI-MS/MS: Application to a Bioequivalence Study, *Scientia Pharmaceutica*, **2010**, 78, 411 - 412.
23. Beckett A.H. and Stenlake J.B. Practical Pharmaceutical Chemistry, Volume II, 4th edition, CBS Publishers and Distributors, New Delhi, **2007,** 284 - 299.
24. Chauhan B., Shubha Rani, Nivsarkar M. and Padh H. A New Liquid - Liquid Extraction method for Determination of Montelukast in Small Volume Human Plasma Samples using HPLC with Fluorescence Detector, *Indian Journal of Pharmaceutical Sciences*, **2006**, 68 (4), 517 - 520.
25. Choudhari V., Kale A., Abnawe S., Kuchekar B., Gawli V. and Patil N. Simultaneous Determination of Montelukast Sodium and Levocetirizine Dihydrochloride in Pharmaceutical Preparations by Ratio Derivative Spectroscopy, *International Journal of Pharm Tech Research*, **2010**, 2 (1), 4 - 9.
26. Code Q2A, Text on Validation of Analytical Procedures. ICH Harmonized Tripartite Guidelines, Geneva, Switzerland, 27th October, **1994,** 1 - 5.

27. Code Q2B, Validation of Analytical Procedures; Methodology. ICH Harmonized Tripartite Guidelines, Geneva, Switzerland, 6th November, **1996**, 1 – 8.

28. Dincer Zafer, Basan Hasan and Goger Nilgun Gunden. Quantitative Determination of Ambroxol in Tablets by Derivative UV Spectrophotometric Method and HPLC, *Journal of Pharmaceutical and Biomedical Analysis*, **2003,** 31(5), 867 - 872.

29. Douglas A. Skoog, James Holler F. and Timothy A. Nieman. Principles of Instrumental Analysis, 5th edition, Thomson Business Information India Pvt. Ltd., New Delhi, **2006,** 331.

30. Eswarudu M.M., Sunil Junapudi and Narendra Chary T. RP-HPLC Method Development and Validation for Simultaneous Estimation of Montelukast Sodium and Levocetirizine Dihydrochloride in Tablet Dosage Form, *International Journal of Pharma World Research*, **2011**, 2 (4), 1 - 18.

31. Fifeld F.W. and Kealey D. Principles and Practice of Analytical Chemistry, 5th edition, Blackwell Publishers, London, **2004,** 5.

32. Grzegorz Bazylak and Luc Nagels J. Simultaneous High - Throughput Determination of Clenbuterol, Ambroxol and Bromohexine in Pharmaceutical Formulations by HPLC with Potentiometric Detection, *Journal of Pharmaceutical and Biomedical Analysis*, **2003**, 32 (4 - 5), 887 - 903.

33. Gupta S.C. and Kapoor V.K. Fundamentals of Mathematical Statistics, 9th edition, Sultan Chand and Sons, New Delhi, **1995**, 2.6, 3.2 - 3.28.

34. Guredeep R Chatwal and Sham K Anand. Instrumental Methods of Chemical Analysis, 5th revised edition, Himalaya Publishing House, Mumbai, **2008,** 2.107 - 2.148.

35. Hadad Ghada M., El-Gindy Alaa and Mahmoud Waleed M.M. HPLC and Chemometrics – assisted UV Spectroscopy Methods for the Simultaneous Determination of Ambroxol and Doxocycline in Capsule, *Spectrochimica Acta Part-A: Molecular and Biomolecular spectroscopy*, **2008**, 70 (3), 655 - 663.
36. Heinanen Maarit and Barabas Coral. Validation of an HPLC Method for the Quantification of Ambroxol Hydrochloride and Benzoic acid in Syrup as Pharmaceutical Form Stress test for Stability Evaluation, *Journal of Pharmaceutical and Biomedical Analysis.* **2001,** 24 (5 - 6), 1005 - 1010.
37. Hobart H.Willard, Lynne L.Meritt,Jr., John A.Dean and Frank A.Settle. Instrumental Methods of Analysis, 7th edition, CBS Publishers and Distributors, New Delhi, **1986**, 169.
38. Hohyun Kim, Jeong-Yeon Yoo, Sang Beom Han, HeeJooLee and Kyung Ryulee. Determination of Ambroxol in Human Plasma using LC-MS/MS, *Journal of Pharmaceutical and Biomedical Analysis*, **2003**, 32(2), 209 - 216.
39. Jain P.S. Stability-Indicating HPTLC Determination of Ambroxol Hydrochloride in Bulk Drug and Pharmaceutical Dosage Form, *Journal of Chromatographic Sciences*, **2010**, 48 (1), 45 - 48.
40. Jan N., Marclay F., Schmutz N., Smith M., Lacoste A., Castella V. and Mangin P. Use of Forensic Investigations in Anti – Doping, *Forensic Science International*, **2011,** 213(1-3), 109 – 113.
41. Joel G. Hardman, Lee E. Limbird and Alfred Goodman Gilman. Goodman and Gillman's. The Pharmacological Basis of Therapeutics, 10th edition, McGraw Hill Medical Publishing Division, New Delhi, **2001,** 741, 1939t, 1984t.

42. Kang Seung Woo, Jang Hae Jong, Moore Victor S., Park Ji-Young, Kim Kyoung-Ah, Youm Jeong-Rok and Han Sang Beom. Enantio Selective Determination of Cetirizine in Human Plasma by Normal – Phase Liquid Chromatography – Atmospheric pressure Chemical Ionization – Tandem Mass Spectrometry, *Journal of Chromatography B*, **2010**, 878 (32), 3351 - 3357.
43. Koh H.L., Yau W.P., Ong P.S. and Hedge A. Current Trends in Modern Pharmaceutical Analysis for Drug Discovery, *Drug Discover Today*, **2003,** 8(19), 889 - 897.
44. Krupa M. Kothekar, Balasundaram Jayakar, Amit P. Khandhar and Rajnish K. Mishra. Quantitative Determination of Levofloxacin and Ambroxol Hydrochloride in Pharmaceutical Dosage Form by Reversed - Phase High Performance Liquid Chromatography, *Eurasian Journal of Analytical Chemistry*, **2007**, 2 (1), 21 - 31.
45. Lakshmana Prabhu S., Shirwaikar A.A., Annie Shirwaikar, Dinesh Kumar C. and Aravind Kumar G. Simultaneous UV Spectrophotometric Estimation of Ambroxol Hydrochloride and Levocetirizine Dihydrochloride, *Indian Journal of Pharmaceutical Sciences*, **2008**, 70 (2), 236 - 238.
46. Mario Thevis, Hans Geyer, Ute Mareck, Gred Sigmund, Jurgen Henke, Lotte Henke and Wilhelm Schanzer. Detection of Manipulation in Doping Control Urine Sample Collection: A Multidisciplinary Approach to Determine Identical Urine Samples, *Analytical and Bioanalytical Chemistry*, **2007,** 388 (7), 1539 - 1543.
47. Mendham J., Denny R.C., Jeffery G.H and Thomas. Vogel's Text book of Quantitative Chemical Analysis, 5th edition, Longman Publishers, UK, **1994,** 10 - 11.
48. Mirza, Shahed, Nanda Rabindra, Dehghan Muhammad Hassan, Nasreen Huda and Feroz Shaikh. Simultaneous Determination of Gatifloxacin and Ambroxol

Hydrochloride from Tablet Dosage Form using Reversed-Phase High Performance Liquid Chromatography, *Chinese Journal of chromatography*, **2008,** 26(3), 358 - 361.

49. Morita M.R., Berton D., Boldin R., Barros F. A. P., Meurer E.C., Amarante A. R., Campos D.R., Calafatti S.A., Pereira R., Abib Jr., E. and Pedrazolli Jr., J. Determination of Levocetirizine in Human Plasma by Liquid Chromatography – Electrospray Tandem Mass Spectrophotometry: Applications to a Bioequivalence Study, *Journal of Chromatography B,* **2008**, 862 (1- 2), 132 - 139.
50. Neela M.Bhatia, Ganbavele S.K., Bhatia M.S., More H.N. and Kokil S.U. RP-HPLC and Spectrophotometric Estimation of Ambroxol Hydrochloride and Cetirizine Hydrochloride in Combined Dosage Form, *Indian Journal of Pharmaceutical Sciences*, **2008**, 70(5), 603 - 608.
51. Patel P.A., Dole M.N., Shedpure P.S. and Sawant S.D. Spectrophotometric Simultaneous Estimation of Salbutamol and Ambroxol in Bulk and Formulation, *Asian Journal of Pharmaceutical and Clinical Research*, **2011**, 4 (3), 42 - 45.
52. Prashanthi N.L., Mohan Ch. Krishna, Manikiran S.S. and Rama Rao N. Estimation of Ambroxol Hydrochloride and Guiaphensin in Tablet Dosage Form by Simultaneous equation method, *International Journal of Research in Ayurveda and Pharmacy*, **2010**, 1 (1), 140 - 146.
53. Priyanka A. Patel, Manjusha N. Dole, Sanjay D. Sawant and Priyanka S.Shedpure. Simultaneous Determination of Salbutamol and Ambroxol in Fixed Dose Combination by Spectrophotometry, *International Journal of Pharmaceutical Sciences and Research*, **2011**, 2 (5), 1225 - 1230.
54. RadhaKrishna T., Narasaraju A., Ramakrishna M. and Satyanarayana A. Simultaneous Determination of Montelukast and Loratidine by HPLC and Derivative

Spectrophotometric Methods, *Journal of Pharmaceutical and Biomedical Analysis*, **2003**, 31 (2), 359 - 368.

55. Rahul P.Gunjal, Raju G., Ramesh babu A., Mallikarjun N., Nalini Shastri and Srinivas R. HPLC and LC-MS Studies on Stress Degradation Behavior of Levocetirizine and Development of a Validated Specific Stability – Indicating Method, *Journal of Liquid Chromatography and Related Technologies*, **2011**, 34 (12), 955 - 965.
56. Rakshit Kanubhai Trivedi, Mukesh C. Patel and Sushant B. Jadhav. A Rapid, Stability Indicating RP-UPLC Method for Simultaneous Determination of Ambroxol Hydrochloride, Cetirizine Hydrochloride and Antimicrobial Preservatives in Liquid Pharmaceutical Formulation, *Scientia Pharmaceutica*, **2011**, 79, 525 - 543.
57. Robert D.Braun, Introduction to Instrumental Analysis, Pharma Book Syndicate, Hyderabad, **2006,**136.
58. Satinsky Dalibor, Huclova Jitka, Ferreira Raquel L.C., Montenegro Maria Conceicao B.S.M. and Solich Petr. Determination of Ambroxol Hydrochloride, methyl Paraben and Benzoic acid in Pharmaceutical Preparations Based on Sequential Injection Technique Coupled with Monolithic Column, *Journal of Pharmaceutical and Biomedical Analysis*, **2006**, 40(2), 287 - 293.
59. Segura J. Is Anti – Doping Analysis so far from Clinical, Legal or Forensic Targets? : The added Value of Close Relationships between Related Disciplines, *Drug Test Analysis*, **2009,** 1(11 -12), 479 – 484.
60. Sethi P.D. HPTLC Quantitative Analysis of Pharmaceutical Formulations, 1st edition, CBS Publishers and Distributors, New Delhi, **1996,** 1 - 18.

61. Shaikh K.A. and Patil A.T. A Stability-Indicating LC Method for the Simultaneous Determination of Levocetirizine Dihydrochloride and Pseudoephedrine Sulfate in Tablet Dosage Forms, *International Journal of Chem Tech Research,* **2010,** 2(1), 454 - 461.

62. Sharma B.K. Instrumental Methods of Chemical Analysis, 25th edition, Goel Publishing House, Meerut, **2006,** S 68 – S 192.

63. Singh R.M., Saini P.K., Mathur S.C., Singh G.N. and Lal B. Development and Validation of a RP-HPLC Method for Estimation of Montelukast Sodium in Bulk and in Tablet Dosage Form, *Indian Journal of Pharmaceutical Sciences*, **2010**, 72 (2), 235 - 237.

64. Smita Sharma, Sharma M.C., Kohli D.V. and Sharma A.D. Development and Validation of TLC-Densitometry Method for Simultaneous Quantification of Montelukast Sodium and Levocetirizine Dihydrochloride Pharmaceutical Solid Dosage form, *Der Pharmacia Lettre,* **2010,** 2 (1), 489 - 494.

65. Sripalakit Pattana, Kongthong Bungon and Saraphanchotiwitthaya Aurasorn. A Simple Bioanalytical Assay for Determination of Montelukast in Human Plasma: Application to a Pharmacokinetic Study, *Journal of Chromatography B*, **2008**, 869 (1- 2), 38 - 44.

66. Su Fenli, Wang Feng, Gao Wei and Li Huande. Determination of Ambroxol in Human Plasma by High Performance Liquid Chromatography - Electrospray Ionization Mass Spectrometry (HPLC-MS/ESI), *Journal of Chromatography B,* **2007**, 853(1 - 2), 364 - 368.

67. Sunil R. Dhaneshwar, Kumudini S. Rasal, Vidhya K. Bhusari, Janaki V. Salunkhae and Amruta L. Suryan. Validated HPTLC Method for Simultaneous Estimation of

Levocetirizine Hydrochloride and Nimesulide in Formulation, *Der Pharmacia Sinica*, **2011**, 2 (4), 117 - 124.

68. Tripathi K.D. Essentials of Medical Pharmacology, 6th edition, Jaypee Publications, New Delhi, **2008,** 143, 196, 205.
69. Umadevi B. and Vetrichelvan T. Development and Validation of UV Spectrophotometric Determination of Doxofylline and Ambroxol Hydrochloride in Bulk and Combined Tablet Formulation, *International Journal of Current Pharmaceutical Research*, **2011**, 3 (4), 89 - 92.
70. Vidhya K. Bhusari and Sunil R. Dhaneshwar. Application of a Stability-Indicating TLC Method for the Quantitative Determination of Levocetirizine in Pharmaceutical Dosage Forms. *International Journal of Advances in Pharmaceutical Sciences*, **2010**, 1, 387 - 394.
71. Wanqun Hu, Yu Xu, Fei Liu, Aixiang Liu and QingxiangGuo. Rapid and Sensitive Liquid Chromatography Tandem Mass Spectrometry Method for the Quantification of Ambroxol in Human Plasma, *Biomedical Chromatography*, **2008**, 22(10), 1108 - 1114.
72. William Kemp. Organic Spectroscopy, 3rd edition, Palgrave Publishers, New York, **2006,** 257-258.
73. Xiao – Lin Zhang, Hong-RongXu, Wei-Li Chen, Nan-Nan Chu, Xue-NingiLi, Gang-Yi Liu and Chen Yu, Matrine Determination and Pharmacokinetics in Human Plasma using LC-MS/MS, *Journal of Chromatography B*, **2009**, 877(27), 3253 - 3256.

74. Yvan Gaillard, Francois Vayssette and Gilbert Pepin .Compared Intrest between Hair Analysis and Urinalysis in Doping controls Results for Amphetamines, Corticosteroids and Anabolic Steroids in Racing cyclists, *Forensic Science International*, **2000,** 107, 361 - 379.